# Sequence Variations in the Ribosomal Decoding and Peptidyl Transferase Sites Determine Sensitivity to Aminoglycosides and Macrolides

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**Dissertation**

**zur**

**Erlangung der naturwissenschaftlichen Doktorwürde**

**(Dr.sc.nat.)**

**vorgelegt der**

**Mathematisch-naturwissenschaftlichen Fakultät**

**der**

**Universität Zürich**

**von**

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**Zürich, 2010**

**To Mom, Dad and Sriskandh**

## Acknowledgements

I owe a great deal to my supervisors, colleagues, friends and members of my family who have been instrumental in the success of this doctoral thesis.

First and foremost I would like to express my sincere gratitude to Prof. Dr. Erik C. Böttger for supporting this thesis work. Erik, it was a pleasure to work in your group and benefit from your knowledge and skills. Thank you for teaching me to ask the right questions and for providing generous resources and possibilities which made this thesis possible.

I am also extremely grateful to Prof. Dr. Leo Eberl for kindly agreeing to co-examine this thesis.

I would like to thank Dr. Sven N. Hobbie and Dr. Christian M. Brüll. Thank you Sven for your limitless patience and for all your sagacious advice during different stages of this thesis. Thank you Christian for your invaluable support in assay development and for all our chats about science and life.

Thanks are also due to Prof. Dr. Peter Sander for his support and for helpful suggestions.

I would like to express my gratitude to Dr. Dmitry Scherbakov and Dr. Rashid Akbergenov. I benefited a lot from their expertise and also from the many fruitful discussions with them.

I am extremely grateful to all of my colleagues at the Institute of Medical Microbiology for providing a stimulating and friendly environment. My special thanks to all the current and former “Ribosomians” Markus, Martin, Mihai, Sarath, Stefan, Tanja Matt, Tanja Janusic. My thanks also to Andreas, Silke Peter, Tobias, Tom, Reto, Agnese, Petra, Caroline, Peter Keller, Silke Polsfuss, Beat and Julianne for creating such a good atmosphere.

I also thank Susanna Salas, Mägi Haug and Dagmar Blatter for their help in various administrative issues.

I am deeply indebted to my “Gang of Zürich” Monica, Aditi, Alok, Amit, Divesh, Geetanjali, Priyanka, Shankar, Kasthuri, Shreya and Vaibhav for all the walks, talks, cooking and movie sessions. Many thanks also to Dr. Neel Sarovar Bhavesh for all his help. Thanks to Manoj and Vasudha for being always by my side despite the distance.

And most importantly thanks to my parents and brother for their unwavering trust, support and encouragement. Their belief in my abilities has been a great source of strength for me throughout my life. It is my desire to dedicate this thesis to them.

# TABLE OF CONTENTS

# I. Summary

Ribosomes are gigantic ribonucleoprotein particles which catalyze the polymerization of amino acids. There is a wealth of genetic and biochemical evidence to support the concept that the rRNA plays a pivotal role in ribosome functions, e.g. mRNA decoding, tRNA binding, peptidyl transfer, subunit association. The ribosome is target for a plethora of antibacterial agents including aminoglycosides, macrolides, ketolides, oxazolidinones, lincosamides. These compounds target different ribosomal functions like peptidyl transfer, mRNA decoding, tRNA translocation. Ribosomal antibiotics mainly bind to the rRNA which typically shows a high degree of phylogenetic sequence conservation within the drug binding pocket. It is unclear whether the minor sequence variations present in the bacterial drug-binding site affect antibiotic susceptibility and/or resistance development. Previously, limitations in genetic manipulation did not allow to study the effect of rRNA sequence variations on bacterial drug susceptibility in isogenic mutants. Rather, investigations were limited to different bacterial species representative of the corresponding sequence variation.

Phylogenetic differences in rRNA provide the basis for selectivity of compounds affecting the ribosome. However, sequence polymorphisms in the mitochondrial small subunit rRNA, namely A1555G and less frequently C1494U, have been identified as primary genetic traits in non-syndromic deafness. Both mutations locate to the A-site of mitochondrial 12S rRNA, a region essential for mRNA decoding. In addition to congenital deafness, the A1555G and C1494U mutations render affected individuals highly susceptible to aminoglycoside induced deafness. The high copy number of mitochondrial DNA in mitochondria and the vast number of mitochondria in a single cell have frustrated any attempt of genetic manipulation of mitochondrial rRNA in lower and higher eukaryotes. The absence of experimental models for diseases associated with mitochondrial rRNA polymorphisms has not only hampered insight into disease pathogenesis but also prevented the rational search for therapeutic interventions.

In the thesis presented here, a derivative of the gram-positive *Mycobacterium smegmatis* rendered single rRNA operon allelic by gene-inactivation techniques was used for the experimental studies. Site-directed mutagenesis of the single chromosomal rRNA operon resulted in cells carrying homogenous populations of mutant ribosomes. Bacterial sequence polymorphisms were introduced representing the different bacterial clades. In addition, nucleotides in 16S rRNA helix 44 of *M. smegmatis* were replaced with those of wild-type and disease-associated mitochondrial versions resulting in hybrid ribosomes with a fully functional eukaryotic rRNA decoding site.

1) The natural sequence variations in the ribosomal peptidyl transfer center of bacteria were found not to affect drug susceptibility, but to impact the resistance phenotype of the A2058G mutation, in particular to the ketolide telithromycin. In contrast, natural sequence variations in the ribosomal A-site of bacteria affected both drug susceptibility and the resistance phenotype of the A1408G mutation.

2) The disease-associated sequence polymorphisms in mitochondrial rRNA, i.e. A1555G and C1494U, significantly reduced the accuracy of translation. Modelling the mitochondrial decoding site on available bacterial X-ray structures allowed to develop a structure-function hypothesis addressing the molecular mechanism of mutation-mediated misreading.

3) Mitochondrial rRNA sequence polymorphisms A1555G and C1494U significantly increased drug binding and ribosomal susceptibility to aminoglycoside-induced inhibition of protein synthesis and aminoglycoside-induced mistranslation.

The results presented in this thesis indicate that misreading of the genetic code is an important molecular mechanism in both mitochondrial rRNA polymorphism-associated deafness and in aminoglycoside-induced ototoxicity. Our data establish the hybrid ribosome approach as an excellent model to study the mechanisms of mutation- and aminoglycoside- mediated dysfunction of the mitochondrial ribosome and to address issues of species-specific drug action in bacteria.

# II. Zusammenfassung

Ribosomen sind riesige Ribonukleoproteinkomplexe, welche die Polymerisation von Aminosäuren katalysieren. Eine Fülle genetischer und biochemischer Untersuchungen weisen darauf hin, dass die rRNA eine entscheidende Rolle bei der Funktion des Ribosoms wie z. B. mRNA-Decodierung, tRNA-Bindung, Peptidyltransfer und Assoziation der Untereinheiten einnimmt. Das Ribosom ist das Angriffsziel für eine Vielzahl von Antibiotika, darunter Aminoglykoside, Makrolide, Ketolide, Oxazolidinone und Lincosamide. Diese Verbindungen greifen in verschiedene ribosomale Funktionen wie Peptidyltransfer, mRNA-Decodierung oder tRNA-Translokation ein. Ribosomale Antibiotika binden hauptsächlich an die rRNA, die innerhalb der jeweiligen Bindungstasche einen hohen Grad an phylogenetischer Sequenzkonservierung aufweist. Es ist unklar, ob die geringen Sequenzvariationen, die in der bakteriellen Wirkstoffbindestelle vorhanden sind, die Empfindlichkeit gegenüber Antibiotika bzw. die Entwicklung von Resistenzen beeinflussen. Bisher stiess man bei genetischen Manipulationen an methodische Grenzen, die es nicht erlaubten den Einfluss bakterieller rRNA-Sequenzvariationen auf die Empfindlichkeit gegenüber Antibiotika in isogenen Mutanten zu analysieren. Untersuchungen waren stattdessen auf verschiedene Bakterienarten begrenzt, die jeweils repräsentativ für die entsprechenden Sequenzvariationen sind.

Phylogenetische Unterschiede in der rRNA bilden die Grundlage für die Selektivität von Antibiotika, die am Ribosom angreifen. Sequenzpolymorphismen in der rRNA der mitochondrialen kleinen Untereinheit, i.e. A1555G und seltener C1494U, wurden als primäre genetische Merkmale nicht-syndromischer Taubheit identifiziert. Beide Mutationen befinden sich in der A-Stelle der mitochondrialen 12S-rRNA, einer zur mRNA-Decodierung notwendigen Region. Zusätzlich zur angeborenen Taubheitsdisposition zeigen die A1555G- und C1494U-Mutationen bei betroffenen Personen eine hohe Anfälligkeit für Aminoglykosid-induzierte Taubheit. Die Multiplizität mitochondrialer DNA-Kopien in Mitochondrien sowie die enorme Anzahl an Mitochondrien in einer einzigen Zelle haben jegliche Versuche zur genetischen Manipulation von mitochondrialer rRNA in niederen und höheren Eukaryonten vereitelt. Der Mangel an experimentellen Modellen für Krankheiten, die mit mitochondrialen rRNA-Polymorphismen assoziiert sind, hat nicht nur den Einblick in die Pathogenese dieser Erkrankungen erschwert, sondern auch die rationale Suche nach therapeutischen Interventionen behindert.

In der vorliegenden Dissertation wurde für die experimentellen Untersuchungen ein Stamm des grampositiven Bakteriums *Mycobacterium smegmatis* verwendet, bei dem durch Geninaktivierungstechniken nur ein einzelnes rRNA-Operon exprimiert wird. Punktgenaue Mutagenese des einzelnen chromosomalen rRNA-Operons führte zu Zellen, die eine homogene Population mutierter Ribosomen aufweisen. Es wurden bakterielle Sequenzvariationen eingeführt, welche die verschiedenen Bakteriengruppen repräsentieren. Darüber hinaus wurden Nukleotide in Helix 44 der 16S-rRNA von *M. smegmatis* mit jenen von wildtyp- und krankheitsassoziierten

mitochondrialen Varianten ersetzt, woraus hybride Ribosomen mit einer vollständig funktionsfähigen eukaryontischen rRNA-Decodierungsstelle resultierten.

1) Es stellte sich heraus, dass die natürlichen Sequenzvariationen im ribosomalen Peptidyltransferasezentrum von Bakterien keine Auswirkung auf die Empfindlichkeit gegenüber Antibiotika haben, aber den Resistenzphänotyp der A2058G-Mutation, insbesondere gegenüber dem Ketolid Telithromycin, beeinflussen. Dagegen wirkten sich natürliche Sequenzvariationen in der ribosomalen A-Stelle von Bakterien sowohl auf die Empfindlichkeit gegenüber Aminoglycosiden als auch auf den Resistenzphänotyp der A1408G-Mutation aus.

2) Die krankheitsassoziierten Sequenzpolymorphismen mitochondrialer rRNA, d. h. A1555G und C1494U, führten zu einer signifikanten Reduktion der Translationsgenauigkeit. Die Modellierung der mitochondrialen Decodierungsstelle anhand vorliegender bakterieller Röntgenstrukturen ermöglichte die Entwicklung einer Struktur-Funktionshypothese, die den molekularen Mechanismus von mutationsvermittelten Lesefehlern beschreibt.

3) Die Sequenzpolymorphismen A1555G und C1494U in mitochondrialer rRNA führten zu einer signifikanten Zunahme der Bindung von Aminoglycosid-Antibiotika. Sie erhöhen die ribosomale Empfindlichkeit gegenüber Aminoglykosid-induzierter Hemmung der Proteinsynthese und Aminoglykosid-induzierten Translationsfehlern.

Die in dieser Dissertation präsentierten Ergebnisse deuten darauf hin, dass Fehler beim Ablesen des genetischen Codes einen wichtigen Mechanismus sowohl bei Taubheit, welche mit Polymorphismen in mitochondrialer rRNA assoziiert ist, als auch bei Aminoglykosid-induzierter Ototoxizität darstellt. Unsere Daten etablieren den Hybridribosomenansatz als ein ausgezeichnetes Modell zur Analyse Mutations- und Aminoglykosid-vermittelter Fehlfunktion des mitochondrialen Ribosoms und zur Untersuchung von Aspekten artspezifischer Antibiotikaempfindlichkeit in Bakterien.

# 1. Introduction

## 1.1 Ribosome: Composition

Ribosomes, the universal riboprotein assemblies, are the nanomachines which translate the genetic code into proteins. These organelles, of a molecular weight of about 2.5 MDa in bacteria and up to 4 MDa in higher organisms, are composed of many different proteins and huge RNA chains accounting for two-thirds of the mass. All ribosomes are constituted of two unequal subunits, which associate during the initiation step of protein biosynthesis. In prokaryotes, the small subunit, denoted as 30S, contains an RNA chain (16S) of about 1500 nucleotides and 20-21 different proteins, whereas the large subunit (called 50S in prokaryotes) has two RNA chains (23S and 5S RNA) of about 3000 nucleotides in total, and 31–35 different proteins (Table 1).

| Characteristics | Bacteria | Archaea | Mitochondria | Eukarya |
|---|---|---|---|---|
| Ribosome size | 70S | 70S | 55S | 80S |
| ***Small subunit*** | | | | |
| Size | 30S | 30S | 28S | 40S |
| Mass (MDa) | 0.8 | 0.8 | 1.2 | 1.4 |
| rRNAs | 16S | 16S | 12S | 18S |
| Number of r-proteins | 20 | 28 | 33 | 32 |
| ***Large subunit*** | | | | |
| Size | 50S | 50S | 39S | 60S |
| Mass (MDa) | 1.6 | 1.6 | 2.4 | 2.6 |
| rRNAs | 23S, 5S | 23S, 5S | 16S | 28S, 5.8S, 5S |
| Number of r-proteins | 34 | 40 | 52 | 46 |

**Table1: Ribosomal composition from the 3 domains of life and mitochondria (42).**

## 1.2 Ribosome: Mechanism of translation

Translation is a multistep process which involves both the ribosomal subunits and a host of translation factors (Figure 1) (46). The mRNA carries the genetic information that is translated by the ribosome and tRNAs bring the amino acids to the ribosome. The ribosome contains three sites for hosting its tRNA substrates, each residing on both the subunits. The A-site hosts aminoacyl-tRNA (aa-tRNA) molecules, while the P-site hosts the growing peptidyl-tRNA (pept-tRNA). E denotes the site of the exiting tRNA. The decoding center resides on the small subunit, hence the mRNA and the tRNA anticodon loops are attached to it, whereas the catalytic site of the ribosome, the peptidyl transferase center (PTC), resides on the large subunit. During the formation of the peptide bond, nucleophilic attack of the amino group of the aa-tRNA in the A site on the carbonyl carbon of pept-tRNA produces a pept-tRNA that is elongated by one amino acid

residue and the deacylated tRNA moves into the E site and then exits the ribosome (34). The pept-tRNA is then translocated into the P-site to facilitate another round of elongation. The decoding and formation of the peptide bonds occur in an iterative manner, resulting in a polypeptide chain with a sequence dictated by the mRNA sequence. All these stages occur usually with the expenditure of energy in the form of guanosine triphosphate (GTP) (53).

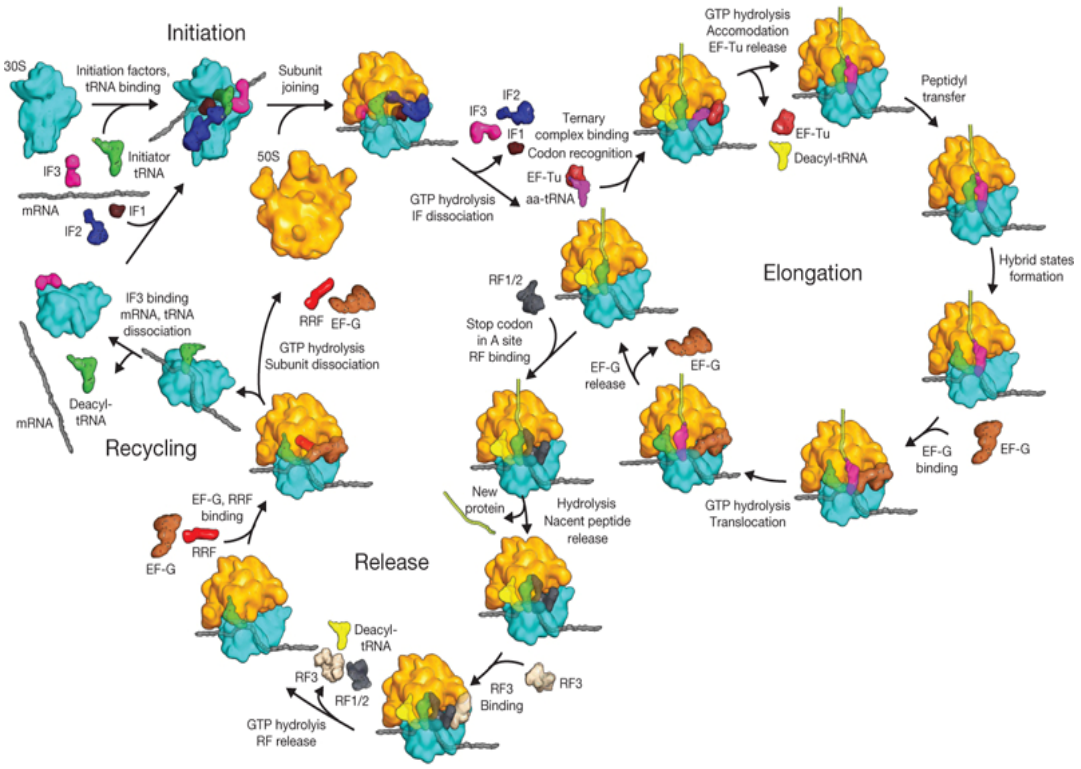



**Figure 1: The different steps of bacterial translation (53)**

Ribosome function has been studied for many decades by biochemical and genetic approaches. These studies have shown that ribosomal RNA (rRNA), the main component of ribosomes, plays a crucial role in essentially all aspects of translation (32). Recent X-ray crystallographic and cryo-EM studies of prokaryotic ribosomes have provided a wealth of structural information about various steps in translation.

tRNA selection is a multistep process that occurs when the anticodon of an aminoacyl-tRNA base pairs with a mRNA codon located in the ribosomal A site. Crystallographic studies have shown that three universally conserved nucleotides of *Escherichia coli* 16S rRNA: G530 from helix 18 and A1492 and A1493 from helix 44 probe the codon-anticodon helix to ensure the accuracy of the codon-anticodon match during translation elongation. When a cognate codon-anticodon interaction occurs, G530 transitions from a *syn* to an *anti* conformation, while A1492 and A1493 flip out into the minor groove of the codon-anticodon helix (35). These conformational changes allow the decoding site to monitor the proper codon-anticodon interaction by hydrogen bonding to 2′ hydroxyl groups on either side of the cognate codon-anticodon helix, providing a sensitive test of codon-anticodon pairing.

Ribosomes catalyze peptide bond formation between aa-tRNA bound to the A site of the ribosome and pept-tRNA at the P site. The active site for peptide bond formation, the PTC, is located on the large (50S) ribosomal subunit. High-resolution crystal structures of the 50S subunit have revealed that the PTC is composed of 23S rRNA (domain V), with no protein within 15 Å of the active site (3). This implies that peptide bond formation is catalyzed by RNA and, thus, the ribosome is a ribozyme. Prior to the PT reaction, the tRNA substrates have to bind their respective sites on the ribosome. The conserved 23S rRNA bases A2451, U2506, U2585, C2452, and A2602 are located at the core of the PTC. The acceptor arms of the A and P site tRNAs are located in a cleft of the 50S interface side. Their universally conserved CCA ends are oriented and held in place by interactions with residues of 23S rRNA near the active site. The peptide bond is formed as a result of nucleophilic attack by the α-amino group of aa-tRNA on the ester carbonyl group of pept-tRNA (4).

Following peptidyl transfer, the P-site tRNA is deacylated and the A-site tRNA has a peptide chain that has one additional residue. To prepare the ribosome for a new round of peptide chain elongation, the tRNAs have to move i.e. the deacylated tRNA needs to be moved from the P-site to the E-site and eventually ejected from the ribosome, while the pept-tRNA has to move from the A-site to the P-site. Moreover, this movement has to be precise, and the reading frame on mRNA has to be preserved. It was proposed that translocation would involve a relative movement of the two subunits, and could occur in separate steps relative to the 50S and 30S subunits in the presence of Elongation Factor G and GTP (17).

The growing polypeptide chain is threaded through a polypeptide exit tunnel which is below the PTC and spans the large ribosomal subunit. It is lined primarily by rRNA but several ribosomal proteins also reach its wall. Recent biochemical findings show that the ribosomal exit tunnel is a dynamic functional entity with the ability to take part in elongation arrest, discrimination and possibly also protein folding (33).

## 1.3 Ribosome: A drug target

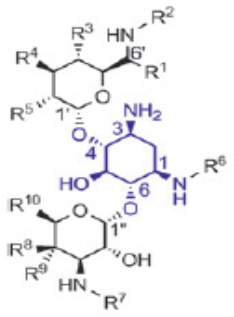
A great variety of natural, semisynthetic or synthetic antibiotics inhibit the growth of bacteria by binding to their ribosomes and interfering with translation (42, 62). For decades, protein synthesis inhibitors have been among the most successful, clinically useful antibiotics (66). The ribosomes are ribozymes because the most important functions are carried out by the rRNA. Thus, the decoding site, the PTC and the protein exit tunnel are formed by rRNAs, and most ribosomal antibiotics interact primarily with these rRNAs (6, 65, 66). More recent crystallographic studies of ribosomal subunits complexed with a variety of antibiotics presented an extended panel of fascinating, detailed understanding of the ribosome–drug interactions at atomic resolution (Table 2).

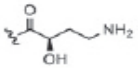
| Drugs targeting the small (30S) ribosomal subunit | |
|---|---|
| **Antibiotic class** | **Mode of action** |
| Aminoglycosides | Bind to the A-site and induce misreading of the genetic code |
| Tetracyclines | Block binding of tRNA to A-site |
| Viomycin, Capreomycin | |
| **Drugs targeting the large (50S) ribosomal subunit** | |
| **Antibiotic class** | **Mode of action** |
| Macrolides and ketolides | Bind to the polypeptide exit tunnel and prevent elongation of nascent peptide |
| Streptogamins | Block peptide bond formation |
| Lincosamides | |
| Pleuromutilins | |

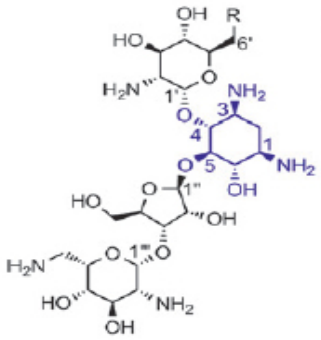
**Table 2: Antibiotics targeting the bacterial ribosome.**

### 1.3.1 Aminoglycosides

In the small ribosomal subunit, the best-studied site of drug action is the decoding centre, which is targeted by aminoglycosides (26). Common aminoglycoside antibiotics share a universal two-ring structure of neamine that includes the 2-deoxystreptamine moiety (ring II). Depending on the type of ring II substitution (4,5 or 4,6), the drugs fall into neomycin/paromomycin or kanamycin/gentamicin families respectively (Figure 2).



| | $R^1$ | $R^2$ | $R^3$ | $R^4$ | $R^5$ | $R^6$ | $R^7$ | $R^8$ | $R^9$ | $R^{10}$ |
|---|---|---|---|---|---|---|---|---|---|---|
| Kanamycin A | H | H | OH | OH | OH | H | H | H | OH | $CH_2OH$ |
| Kanamycin B | H | H | OH | OH | $NH_2$ | H | H | H | OH | $CH_2OH$ |
| Tobramycin | H | H | OH | H | $NH_2$ | H | H | H | OH | $CH_2OH$ |
| Gentamicin C1 | $CH_3$ | $CH_3$ | H | H | $NH_2$ | H | $CH_3$ | OH | $CH_3$ | H |
| Gentamicin C2 | $CH_3$ | H | H | H | $NH_2$ | H | $CH_3$ | OH | $CH_3$ | H |
| Gentamicin C1a | H | H | H | H | $NH_2$ | H | $CH_3$ | OH | $CH_3$ | H |
| Amikacin | H | H | OH | OH | OH |  | H | H | OH | $CH_2OH$ |



| | R |
|---|---|
| Paromomycin | OH |
| Neomycin B | $NH_2$ |

**Figure 2: Chemical structures of 2-deoxystreptamine (ring II)-containing aminoglycosides. The 2-DOS scaffold is highlighted in blue.**

The decoding site comprises a segment of an extended rRNA helix 44 that resides on the interface surface of the small subunit (Figure 3a) (35). Both structural and functional studies indicate that the degree of aminoglycoside binding to the prokaryotic decoding site is greatly influenced by two nonconserved nucleotides, A1408 and G1491 (*E. coli* numbering used throughout) (39, 40). A1408 makes hydrogen bonds with the group at the 6′ position of ring I of aminoglycosides to form a pseudo base-pair interaction (61). G1491 provides a stacking interaction with ring I to stabilize the pseudo base-pair interaction between ring I and A1408 (Figure 3b) (39). The universal interactions of rings I and II of the neamine moiety of aminoglycosides with the decoding centre are supplemented by opportunistic ('fuzzy') interactions that drug-specific rings III and IV of aminoglycosides establish with helix 44 of 16S rRNA (26-28). These fuzzy contacts fine tune placement of the drug within its RNA target and contribute to consequence of aminoglycoside resistance mutations.

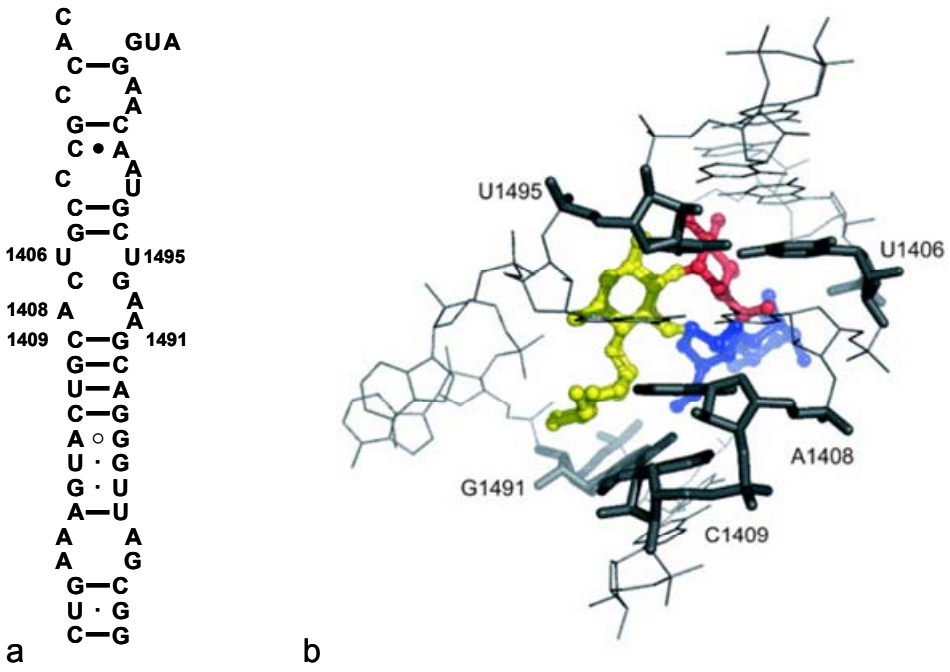



**Figure 3: (a) Secondary structure of helix 44 of the 16S rRNA. (b) View of the three-dimensional structure of the A-site complexed with 4,5- and 4,6-disubstituted 2-deoxystreptamines: the common neamine core is denoted in yellow; ring III of the 4,6-compounds (tobramycin) is denoted in red; and rings III and IV of the 4,5-compounds (paromomycin) are denoted in blue. Key nucleotides (*E. coli* numbering) of the binding site are given in bold (7).**

Ribosome crystallography provided a clear view of the mechanism of action of aminoglycoside drugs that act at the decoding centre. The neamine core-based aminoglycoside drugs affect the ribosome accuracy at the initial step of aminoacyl-tRNA selection (11). During decoding, the ribosome monitors the codon-anticodon interaction to ensure that the aa-tRNA is cognate to the mRNA. This function involves two universally conserved nucleotides of the 16S rRNA, A1492 and A1493, which flip-out of helix 44 in the 30S subunit to monitor the minor groove of the codon-anticodon duplex (Figure 4). Presumably the energy required to flip-out A1492 and A1493 during decoding is compensated for by additional A-minor interactions established with the codon-

anticodon duplex, thus stabilizing this “flipped out” conformation (35). This switch occurs during mRNA decoding when these two adenines monitor the accuracy of codon-anticodon base pairing at the first and second anticodon positions. Flipping out of the adenines 1492/1493 during decoding signals the establishment of an accurate codon-anticodon pairing. In the presence of near-cognate tRNA these compensatory interactions are presumably insufficient to stabilize the flipped out A1492 and A1493 and thus the near-cognate tRNA dissociates. Binding of the universal aminoglycoside rings I and II to the decoding centre changes the orientation of two universally conserved adenine residues at positions 1492 and 1493 of 16S rRNA (Figure 4). Reorientation of A1492/A1493 induced by aminoglycosides explains the miscoding effect of these drugs: in the presence of a bound aminoglycoside, the decoding centre issues an approval signal, even on the binding of a near-cognate tRNA, thereby increasing the frequency of amino acid misincorporation (36).

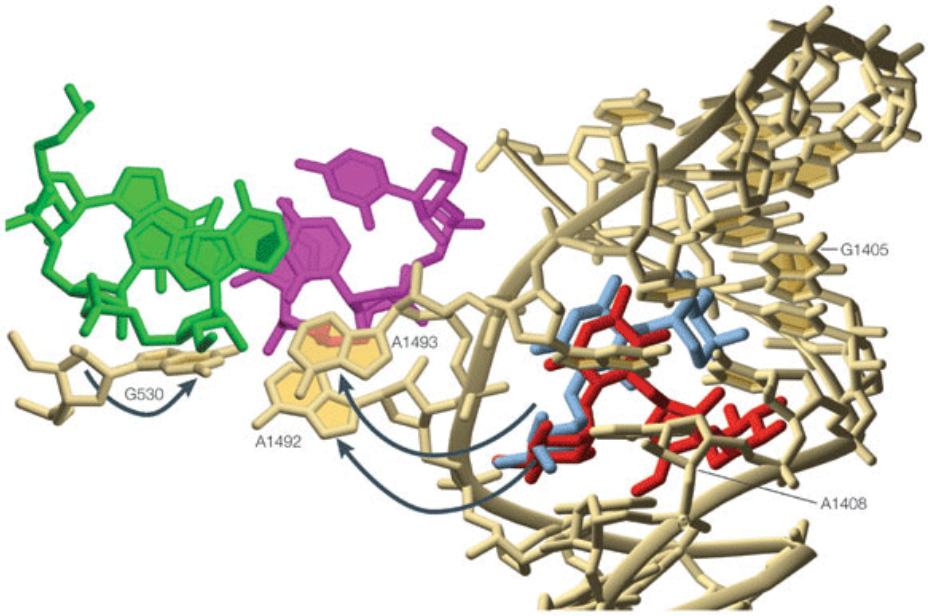



**Figure 4: Nucleotides A1492 and A1493 are shown in the conformation that they adopt after having flipped out of the 16S rRNA helix 44 to interact with an mRNA codon (magenta) and its cognate tRNA anticodon (green) at the ribosomal aminoacyl site. The conformational changes induced at these nucleotides and at G530 are indicated by the arrows. The aminoglycoside antibiotics paromomycin (red) and geneticin (blue) bind within helix 44 as shown, and induce a similar, but not identical, conformational shift in A1492 and A1493. Adapted from (42).**

### 1.3.2 Macrolides and ketolides

Amongst the many antibiotics that target the large (50S) ribosomal subunit the macrolides are the most well studied. Macrolides are polyketide compounds synthesized by the actinomycetes and can be classified structurally into groups in a variety of different ways easiest being on the basis of the size of their macrolactone ring, which can vary significantly from as small as 8-12 membered (methylmycin) to as large as 20 (rapamycin)(47). However, the most commonly used macrolides consist of a 14- to 16-atom lactone ring decorated with one or several sugars and side-chains (Figure 5). The emergence of bacterial strains resistant to macrolides has resulted in

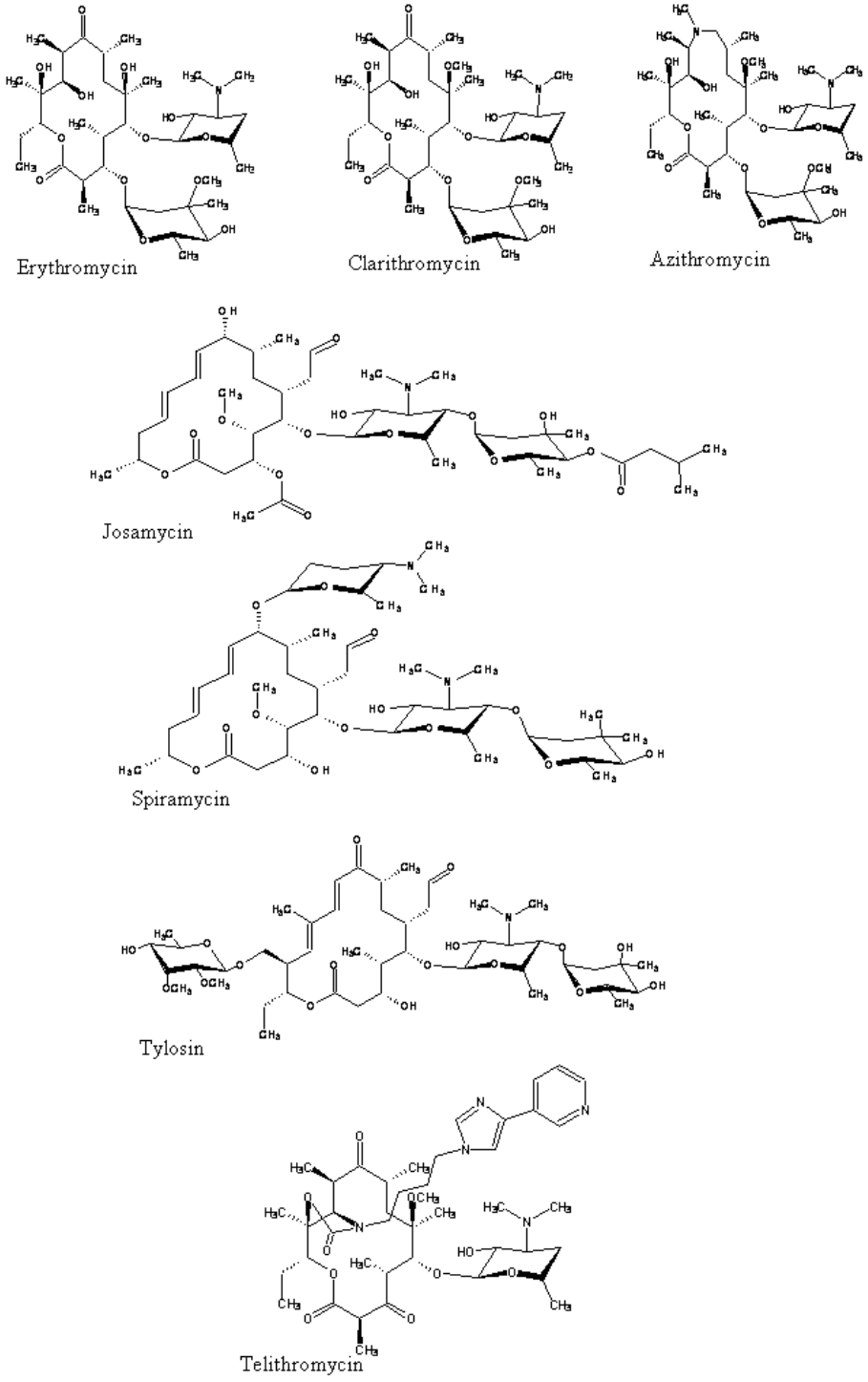



**Figure 5: Structures of commonly used macrolides: 14-membered erythromycin and clarithromycin, 15-membered azithromycin, 16-membered spiramycin. tylosin and josamycin and the ketolide telithromycin.**

the introduction of ketolides. Ketolides are semi-synthetic derivatives of macrolides and possess additional side-chains and modification such as the alkyl-aryl side chain of telithromycin (10). These drugs bind at the upper segment of the nascent peptide exit tunnel in the large ribosomal subunit near the peptidyl transferase centre (52, 59).

Crystallographic studies of macrolides complexed to the large ribosomal subunit showed a conserved interaction of the macrolide core with the ribosome. The lactone ring binds to the wall of the tunnel primarily because of the hydrophobic interactions that involve, among others, residues A2058 and A2059 (Figure 6) (23).

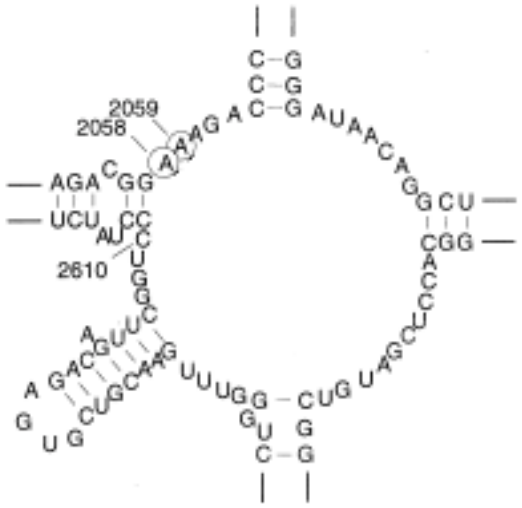



**Figure 6: Secondary structure of domain V of 23S rRNA. rRNA residues are numbered according to their homologous positions in *E. coli* 23S rRNA.**

The C5-linked sugars project toward the PTC. Their hydrophobic and hydrogen bonding interactions with the 23S rRNA residues A2058 and A2059 contribute significantly to the binding energy of the drug (Figure 7). Contacts of the macrolactone ring and the C5-linked sugar residues

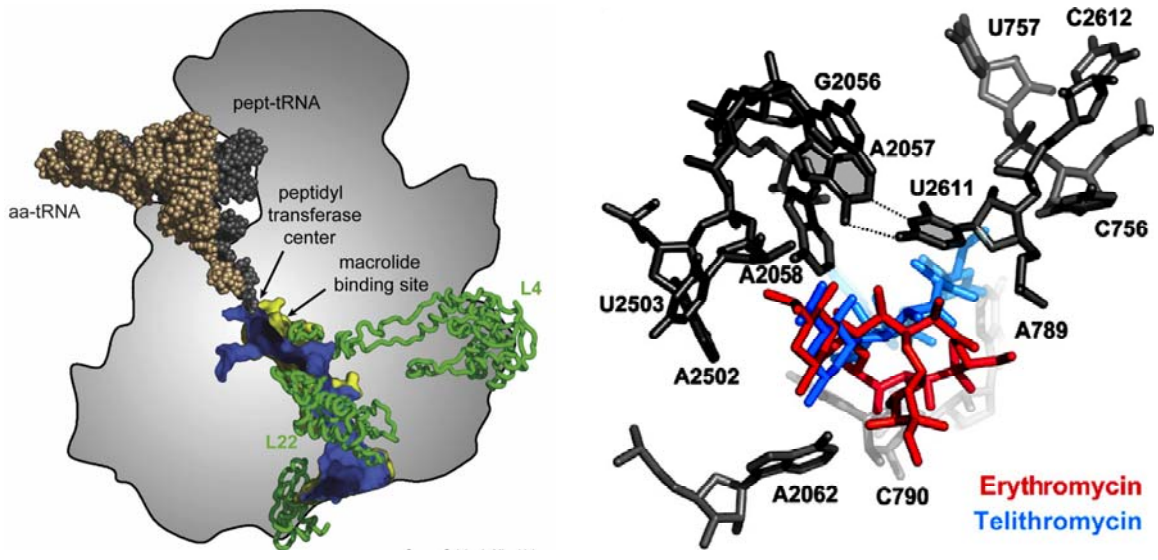



**Figure 7: Binding of macrolides to the large ribosomal subunit. (a) Macrolide binding pocket located near the peptidyl transferase center. aa-tRNA (brown), pept-tRNA (black) and ribosomal proteins L4 and L22 (green), lining the polypeptide exit tunnel (blue) are indicated (31). (b) A view into the macrolide binding pocket. The positions of erythromycin (red) and telithromycin (blue) are superposed (38).**

involve exclusively rRNA and account for a significant portion of the drugs' binding energy. Other sugar residues found in some of the macrolide antibiotics make additional contacts with the ribosome target. For example, the C14-linked mycinose residue of tylosin protrudes down the tunnel, away from the PTC and interacts with the loop of helix 35 in domain II of 23S rRNA and with ribosomal protein L22 (37). The extended alkyl–aryl side-chain of clinically relevant ketolides bound to the bacterial ribosome appears to project in the same direction and probably makes similar contacts (5). These latter drug-specific contacts may contribute significantly to the drug affinity for its ribosomal binding site (24). Bound to the bacterial (*Deinococcus radiodurans*) large subunit (D50S), the sidechain of telithromycin penetrates deeper into the tunnel forming stacking interactions with A752 (5), consistent with the protection of A752 from chemical modification (13). Interestingly, in contrast to the similar positions of the lactone rings, the conformation of the telithromycin side-chain bound to the archaeal (*Haloarcula marismortui*) large subunit (H50S) is dramatically different. These structural differences that modulate the mode of binding of the drug to the ribosome probably stem from the low sequence conservation within this region. More generally, this suggests that antibiotic binding to ribosomes of different organisms/strains may utilize distinct binding modes, which has important implications for structure-based drug design (63). Such variation in binding modes is also illustrated by the binding of two molecules of azithromycin to *D. radiodurans* 50S ribosomes (51), but only one molecule to *H. marismortui* ribosomes (23).

Significant progress has also been achieved in understanding the mode of action of macrolides. The binding of macrolides in the exit tunnel close to the peptidyl transferase centre inhibits translation by blocking progression of the nascent peptide through the exit tunnel (52). The distance between the PTC, where formation of peptide bonds takes place and the macrolide-binding site is only 10 Å. A three to four amino acid long nascent peptide should reach the bound antibiotic. Yet, accurate measurements showed that pept-tRNA dropped-off of the erythromycin-bound ribosome carried nascent peptides which were six to eight amino acids long (55). Ribosomes with bound telithromycin could polymerize even longer peptides 9–10 amino acid residues long before the dissociation of pept-tRNA (55). The effect of macrolides in blocking egression of the nascent polypeptide chain may not be uniform for all mRNAs, but rather be dependent on the nascent polypeptide chain being synthesized (31). Indeed, macrolides with extensive side-chains extending from the C5 position even inhibit peptidyl transferase activity to varying extents, such as carbomycin (100% inhibition), spiramycin (85%) and tylosin (~60%), whereas those with shorter side-chains, such as erythromycin, do not (43).

## 1.4 Natural rRNA sequence variations and drug susceptibility

Protein synthesis is a key and universal process, and the high evolutionary conservation of functional sites within rRNAs, targeted by ribosomal drugs, implies limitations with respect to selectivity and toxicity (8). Still, comparisons of rRNA sequences have shown subtle differences in these locations, despite their conservation, and these minute differences might govern drug

selectivity (1). Thus, a single nucleotide can determine the selectivity of drugs affecting protein synthesis. However, a multitude of different nucleotides located within the rRNA participate in the binding of a drug to its target region; correspondingly, several different nucleotide substitutions can be associated with drug selectivity (6, 8). As a result, the same antibiotic might bind in different modes to slightly different ribosomal pockets, and this can be influenced not only by the often-conserved nucleotides of a functional site targeted by an antibiotic but also by the less-conserved peripheral rRNA residues (56).

Nucleotides of 16S rRNA helices 18, 34 and 44 form the A-site and are highly conserved, reflecting their importance in mRNA decoding (21). While aminoglycosides form a number of hydrogen bonds with different nucleotides in helix 44, their interactions with rRNA residues 1408, 1409 and 1491 appear to be most critical for drug binding (27, 28, 39). Minor sequence variations occur within the aminoglycoside-binding pocket of helix 44 involving 16S-rRNA base-pair 1409-1491 (21). The majority of eubacteria are characterized by a 1409 pyrimidine-1491 purine (C-G) interaction whereas propionibacteria carry a 1409 purine-1491 pyrimidine (A-U) base pair (Figure 8). 16S rRNA residue 1408 is an adenine in all bacteria. Among all A-site mutations that confer aminoglycoside resistance, the 1408 adenine to guanine mutation is the predominant alteration (44, 48). It is also believed to function as the main specificity determinant of aminoglycosides, because higher eukaryotes carry a guanine at this position (8).

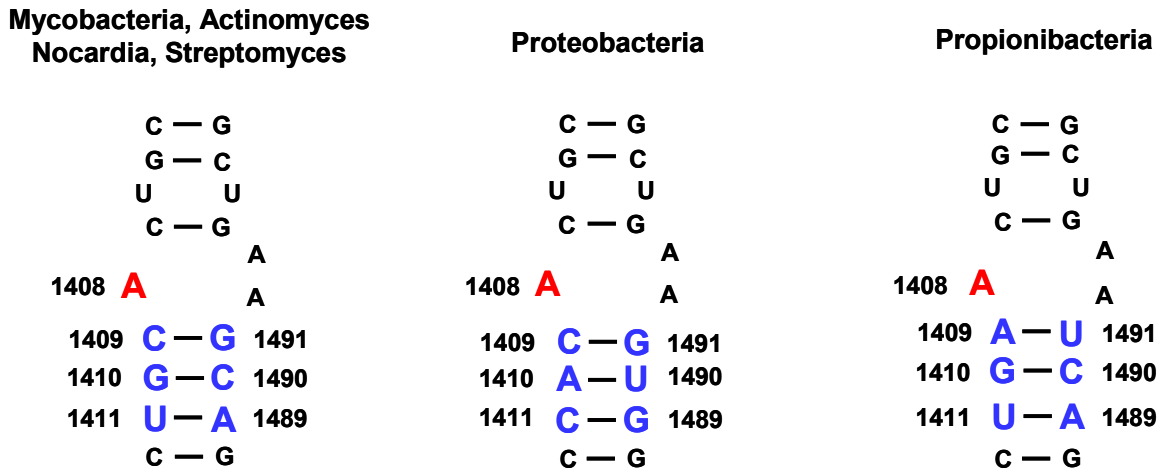



**Figure 8: Secondary structure of 16S rRNA helix 44 decoding site. rRNA residues are numbered according to their homologous positions in *E. coli* 16S rRNA. Phylogenetic sequence variations analyzed in this study are represented in blue, the adenine at position 1408 in red.**

Most rRNA nucleotides in the inner peptidyl-transferase region of the large ribosomal subunit are phylogenetically conserved (22). The macrolide/ketolide family of antibiotics binds to a hydrophobic cleft formed by residues 2058, 2059 and 2611 in domain V of 23S rRNA (24). The adenines at 23S rRNA positions 2058 and 2059 are phylogenetically conserved in bacteria and play an important role in binding, selectivity and resistance towards macrolides/ketolides (1, 41, 49) (Figure 9). Ribosomal susceptibility to macrolides and ketolides is also determined by proper

Watson-Crick base pairing between nucleotides at positions 2057 and 2611 (12), which are typically G-C in proteobacteria and A-U in mycobacteria.

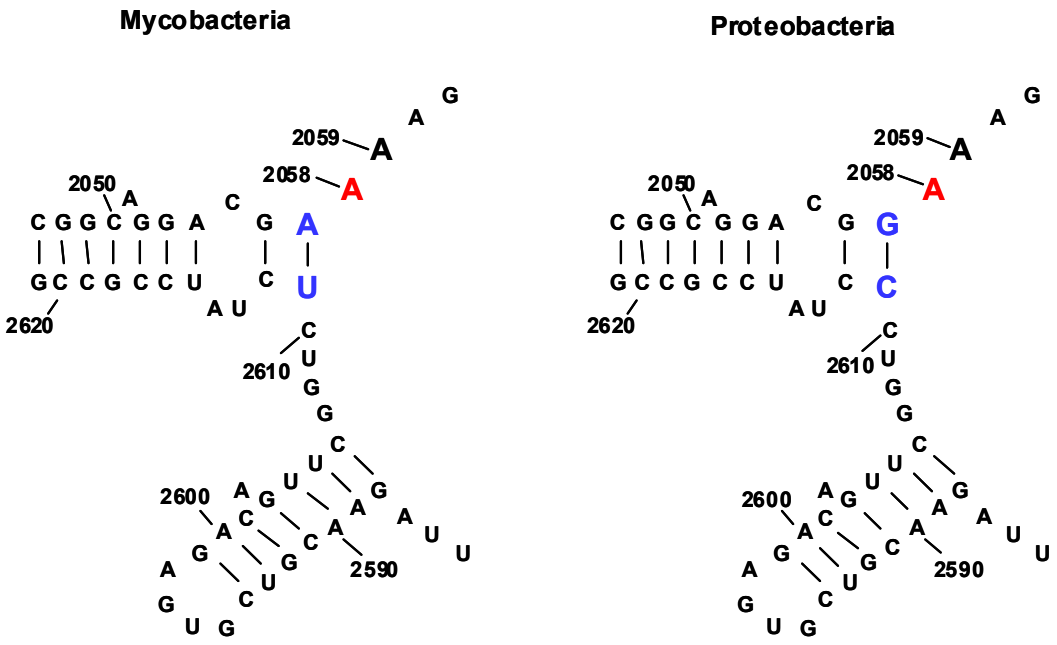



**Figure 9: Secondary structure of domain V of the 23S rRNA. rRNA residues are numbered according to their homologous positions in *E. coli* 23S rRNA. Base pair 2057-2611 is represented in blue and the adenine at position 2058 in red.**

## 1.5 Mitochondrial organization and translation

Mitochondria are intracellular double membrane-bound structures found ubiquitously in eukaryotes and are essential for survival. Their primary function is to support aerobic respiration and to provide energy substrates (such as ATP) for intracellular metabolic pathways. Mitochondria have also been shown to play an important role in cell signalling, particularly in signalling for apoptotic cell death. Mitochondria host several metabolic pathways, including the Krebs cycle, β-oxidation, and lipid and cholesterol synthesis. Given their fundamental role in the human body, defects of mitochondrial function can have disastrous consequences.

Mitochondria are the only location of extra-chromosomal DNA within the vertebrate cell, and they are under the dual genetic control of both nuclear DNA and the mitochondrial genome. Human mtDNA is extremely compact and contains no non-coding sequences. The mitochondrial genome consists of a multicopy, circular dsDNA molecule (16.6 kb in humans), which encodes 13 essential polypeptides of the OXPHOS system and the necessary RNA machinery (2 rRNAs and 22 tRNAs) for their translation within the organelle. The remaining protein subunits that make up the respiratory-chain complexes, together with those required for mtDNA maintenance, are nuclear-encoded, synthesized on cytoplasmic ribosomes, and are specifically targeted and sorted to their correct location within the organelle. MtDNA is highly polymorphic, with several differences in sequence between individuals from the same ethnic group and more between

those in different groups. MtDNA haplotypes are based upon specific patterns of polymorphisms and seem to influence the ageing process, susceptibility to some diseases, and the expression of some mtDNA mutations. All 13 proteins encoded by human mtDNA are subunits of the respiratory chain and OXPHOS (Figure 10).

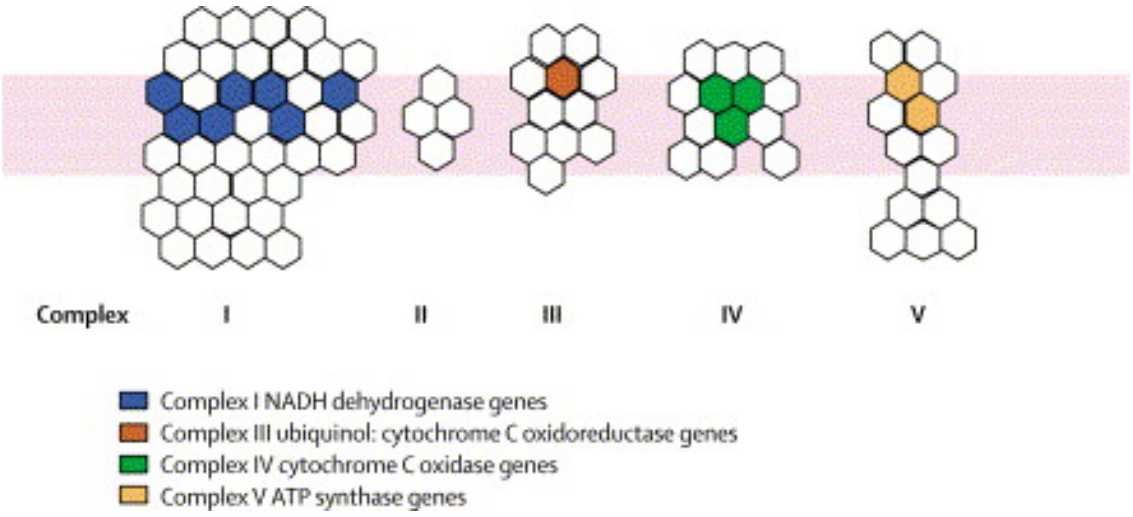



**Figure 10: Mitochondrial respiratory chain and oxidative phosphorylation system. Each hexagon represents a polypeptide product of a single gene (50).**

Mitochondrial genetics is different from Mendelian genetics in almost every aspect, from the uniparental inheritance of disease mutations, to the presence of many copies of the genome within a single cell and the basic mechanisms that underlie replication and control of transcription. The polyploid nature of the mitochondrial genome up to several thousand copies per cell gives rise to an important feature of mitochondrial genetics, homoplasmy and heteroplasmy. In simple terms, homoplasmy is when all copies of the mitochondrial genome are identical; heteroplasmy is when there is a mixture of two or more mitochondrial genotypes. Some mutations affect all copies of the mitochondrial genome (homoplasmic mutation), whereas others are only present in some copies of the mitochondrial genome (heteroplasmic mutation). The differences between the two genetic systems in human cells are probably a relic of evolution, but lead to some fascinating biology that dictates the functional consequences of mtDNA mutations.

The mRNAs for the 13 mtDNA-encoded OXPHOS proteins are translated on mitochondrial ribosomes. Human 55S mitoribosomes contain two subunits, i.e. 28S and 39S. The small ribosomal subunit comprises 12S rRNA and 28 proteins, and the large subunit contains the 16S rRNA and 48 proteins. Surprisingly, the rRNA:protein ratio in mitoribosomes is different (1:2) to that in cytosolic and bacterial ribosomes (2:1). Mitoribosomes contain a higher protein content and lack several of the major rRNA structures of bacterial ribosomes.

## 1.6 Diseases caused by mitochondrial dysfunction

Mitochondrial defects can be inherited or acquired. Inherited mtDNA mutations are responsible for many clinical abnormalities, including various forms of neuropathy, myopathy, cardiomyopathy,

retinal degeneration, diabetes mellitus and sensorineural hearing loss (SNHL) (18). In addition, there is increasing evidence that acquired mtDNA mutations are involved in aging and age-related diseases such as cancer, and diabetes (54). Healthy individuals appear to have only a single mtDNA genotype (i.e., homoplasmic), but, in many mitochondrial disease states, there are mixed mtDNA genotypes (i.e., heteroplasmic). The amount of heteroplasmy varies from tissue to tissue, and for cells within a tissue. Inherited deafness-associated mtDNA mutations usually occur in the genes encoding proteins or genes for components of the protein-synthesizing apparatus, i.e. rRNAs and tRNAs.

## 1.7 Mitochondrial 12S rRNA mutations and sensorineural hearing loss

Mutations in mitochondrial DNA (mtDNA) have been found to be associated with both aminoglycoside-induced and non-syndromic deafness (16, 60). Mitochondrial rRNA mutations associated with SNHL are only found in the 12S rRNA gene, i.e. A1555G (14, 45), C1494T (67), T1095C (57, 58), A827G (64), and 961 mutations (2). In particular, the A1555G mutation has been found to be responsible for nonsyndromic deafness in many families of different ethnic backgrounds (15). In the absence of exposure to aminoglycosides, the A1555G mutation produces a clinical phenotype that varies considerably among family members, ranging from severe congenital deafness to moderate progressive hearing loss of later onset to completely normal hearing (14, 30). Affected persons are homoplasmic for the mutation, a condition that appears to be more frequent in certain populations. Functional characterization in an Arab-Israeli family demonstrated that more severe biochemical defects were observed in mutant lymphoblastoid cell lines derived from symptomatic individuals than in cell lines derived from asymptomatic individuals (19). However, under a constant nuclear background, a nearly identical degree of mitochondrial dysfunction was observed in cybrid cell lines derived from symptomatic or asymptomatic individuals from this family (20). These genetic and biochemical data strongly point out that the A1555G mutation is a primary factor underlying the development of deafness and other factors play a role in modulating the phenotypic expression of the hearing loss associated with the A1555G mutation. Clinical, genetic, and biochemical data have shown that the aminoglycoside antibiotics (45), mitochondrial haplotypes (30), and nuclear modifier genes (20) are three major modulators for the phenotypic expression of the deafness-associated 12S rRNA mutations. Although hearing loss caused by A1555G mutation is usually nonsyndromic, additional symptoms including Parkinson disease and cardiomyopathy have been reported on occasions (60).

## 1.8 Aminoglycosides and ototoxicity

Mitochondrial ribosomes share more similarities with bacterial ribosomes than do cytosolic ribosomes (29). Therefore, it is reasonable to suggest that the ototoxic site of action of aminoglycoside antibiotics involves the mitochondrial ribosome. There are two forms of aminoglycoside-induced deafness: sporadic dose-dependent and inherited idiosyncratic. The fact

that aminoglycoside hypersensitivity is often maternally transmitted suggests that mtDNA mutation(s) are involved in aminoglycoside ototoxicity (16). Sequence analyses of the mitochondrial genome in patients with aminoglycoside ototoxicity have led to the identification of ototoxic mtDNA mutations in the 12S rRNA gene: A1555G, and C1494T mutations (Figure 11). These two mutation account for ototoxicity in 17-33% of the cases (15).

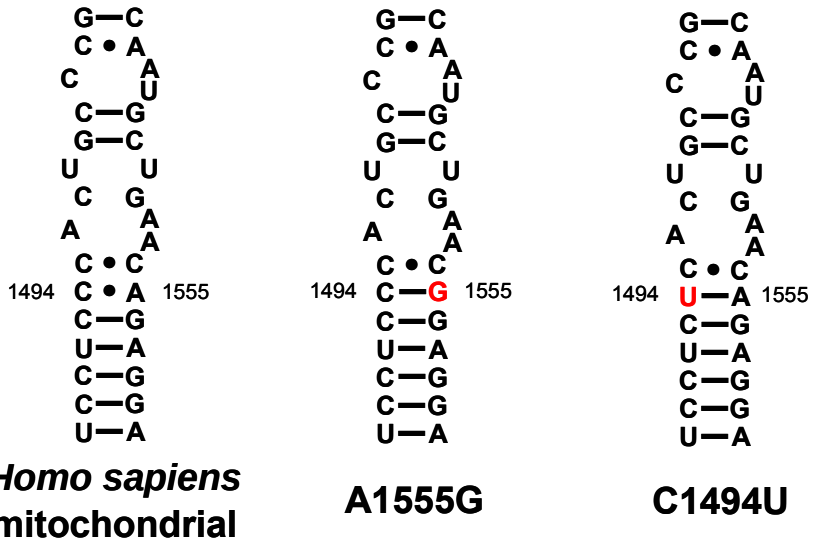



**Figure 11: Secondary structure of wild-type helix44 nucleotides of human mitochondrial 12S rRNA in comparison to disease-associated mutations.**

The A1555G mutation has been found in a number of families and sporadic patients with aminoglycoside-induced severe hearing loss (14). The A1555G mutation is located at a highly conserved region of 12S rRNA that is an essential part of the decoding site of the small ribosomal subunit and important for the action of aminoglycosides. In humans, the nucleotide at position 1555 in the 12S rRNA gene in wild-type cells is A, and, when A is mutated to G, it would pair with C at position 1494 (18). In addition to clinical variability in the phenotypic expression of aminoglycoside ototoxicity, there is also some evidence that the ototoxicity in patients is restricted to the cochlea and the vestibular systems remain unaffected. Patients with hearing loss after treatment with streptomycin did not reveal any vestibular abnormality (9). This tissue specificity maybe because aminoglycosides are concentrated within cochlear cells and remain there for prolonged periods (25).

Another mitochondrial mutation predisposing to aminoglycoside ototoxicity was identified in a large Chinese pedigree with maternally-inherited hearing loss (67). Family members in the maternal line demonstrated hearing ranging from severe hearing loss to normal hearing, and had subsequent severe to profound hearing loss when exposed to aminoglycosides. Mutational analysis of the mitochondrial 12S rRNA revealed a homoplasmic C to U transition at position 1494. Interestingly this mutation leads to a nucleotide pairing U1494-A1555 in the penultimate loop of the 12S rRNA in the conserved aminoglycoside binding region very similar to the base pairing generated by the A1555G mutation C1494-G1555. In lymphoblastoid cell lines derived

from individuals carrying the C1494T mutation, exposure to a high concentration of paromomycin or neomycin caused a variable but significant increase in doubling time, as well as a significant decrease in the rate of total oxygen consumption as compared with controls (68). These results suggest that the C1494T mutation is a primary factor related to hearing loss and that the nuclear genetic background may play a role in the phenotypic expression associated with this mutation.

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# Mitochondrial deafness alleles confer misreading of the genetic code

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Edited by V. Ramakrishnan, Medical Research Council, Cambridge, United Kingdom, and approved January 14, 2008 (received for review August 2, 2007)

**Despite the fact that important genetic diseases are caused by mutant mitochondrial ribosomes, the molecular mechanisms by which such ribosomes result in a clinical phenotype remain largely unknown. The absence of experimental models for mitochondrial diseases has also prevented the rational search for therapeutic interventions. Here, we report on the construction of bacterial hybrid ribosomes that contain various versions of the mitochondrial decoding region of ribosomal RNA. We show that the pathogenic mutations A1555G and C1494T decrease the accuracy of translation and render the ribosomal decoding site hypersusceptible to aminoglycoside antibiotics. This finding suggests misreading of the genetic code as an important molecular mechanism in disease pathogenesis.**

decoding | mitochondria | mutant rRNA | ribosomes | disease

Genes encoded by mitochondrial DNA (mtDNA) have been linked to a variety of diseases (1, 2). One of the most common phenotypes associated with mitochondrial diseases is sensorineural deafness; corresponding mitochondrial mutations localize to tRNA and rRNA genes (2, 3). In particular, the single-nucleotide alteration A1555G has been identified as a major source of nonsyndromic deafness (4) and has since been found in patients from all ethnic backgrounds and geographic origins. More recently, a C1494T point mutation has been associated with nonsyndromic deafness (5); C1494 forms a noncanonical RNA base pair with the adenine encoded by 12S rRNA position 1555 (Fig. 1). Both mutations locate to the penultimate helix of mitochondrial 12S rRNA, which is a component of the aminoacyl-tRNA acceptor site (A site), a region of the small subunit rRNA essential for mRNA decoding (6). In bacteria, the A site is a target for aminoglycoside antibiotics, compounds that are compromised by a substantial degree of ototoxicity (7). Presumably, drug toxicity relates, at least in part, to limited selectivity between the bacterial and the mitochondrial A site and to the mechanism of drug action, i.e., miscoding (8). Interestingly, and in addition to congenital deafness, the A1555G and the C1494T mutations render affected individuals highly susceptible to aminoglycoside-induced deafness (4, 5).

By itself, the A1555G mutation produces a clinical phenotype that may range from severe congenital deafness through moderate progressive hearing loss of later onset to normal hearing (3). Evidence has accumulated that the nuclear background plays an important role in the phenotypic manifestation of nonsyndromic deafness associated with mitochondrial rRNA mutations (9, 10), although cochlear alterations have been demonstrated to be present in symptomatic and asymptomatic carriers of the A1555G mutation (11). Biochemical investigations of deafness-associated mtDNA mutations have so far relied on the characterization of transmitochondrial cell lines, constructed by transferring mitochondria from cell lines of affected patients into the human cell line p°206 lacking mtDNA (9, 10). Nonetheless, the mechanistic link between mitochondrial mutation and disease has remained largely elusive and is still to be established, mainly because of the absence of suitable experimental models. Mice models for mitochondrial diseases have been limited to nuclear-encoded proteins, as the technical hurdles to create animal models of pathogenic mtDNA mutations remain formidable (12). In the absence of experimental models for ribosomes of higher eukaryotes, a spontaneous yeast mutant with a mitochondrial rRNA C1477-to-G alteration conferring resistance to paromomycin has been used as a model for the human mitochondrial A1555G deafness mutation (13). However, this yeast mutant does not show a discernible phenotype in translation (13, 14), and yeast mitochondrial rRNA position 1477 corresponds to human mitochondrial rRNA position 1493 rather than 1494 [see supporting information (SI) Fig. 5 and compare with Fig. 1]. We wanted to test whether the prokaryotic A site in the bacterial ribosome can be modified so as to serve as a model for the A site of mitochondrial ribosomes, as it would allow the study of the pathogenesis of mitochondrial rRNA-associated deafness mutations.

## Results and Discussion

Much of the small subunit A-site rRNA, i.e., the corresponding regions of helices 18, 34, and 44 of 16S rRNA, is highly conserved in structure and nucleotide sequence between bacteria and mitochondria (15). Toward this end, we constructed bacterial hybrid ribosomes comprising the mitochondrial homologue of bacterial helix 44 (H44) of the 16S RNA decoding region by using a single-rRNA allelic derivative of *Mycobacterium smegmatis* and a previously described mutagenesis strategy (16). After several attempts to transfer parts of the mitochondrial H44 into *M. smegmatis* ribosomes, we were eventually able to generate recombinant strains with homogenous populations of hybrid ribosomes in which the central 34-nt part of rRNA helix 44 is identical to that of the human mitochondrial decoding site (Fig. 1). We next constructed recombinants where the bacterial H44 is replaced with mitochondrial deafness alleles corresponding to mtDNA mutations A1555G and C1494T. The resulting mutant mitochondrial hybrid ribosomes differ from the wild-type mitochondrial hybrid only in nucleotide position 1555 or 1494, corresponding to *Escherichia coli* positions 1490 and 1410 (Fig. 1).

Because both mitochondrial rRNA mutations localize within the ribosomal decoding site, we hypothesized that they may affect the rate or accuracy of protein synthesis. For a biochemical analysis of the deafness mutations and their effect on mRNA decoding and polypeptide synthesis, 70S hybrid ribosomes were purified and studied in a cell-free translation assay with an AUG$(UUU)_{12}$ mRNA as template. This template allows the determination of both the rate of amino acid incorporation and the frequency of codon misreading; codon ambiguity between leucine and phenylalanine is at the third codon position, UUU/

Author contributions: S.N.H. and C.M.B. contributed equally to this work; S.N.H. and E.C.B. designed research; S.N.H., C.M.B., S.A., S.K.K., D.S., and E.C.B. performed research; S.N.H., C.M.B., S.A., S.K.K., D.S., and E.C.B. analyzed data; and S.N.H. and E.C.B. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0707265105/DC1.

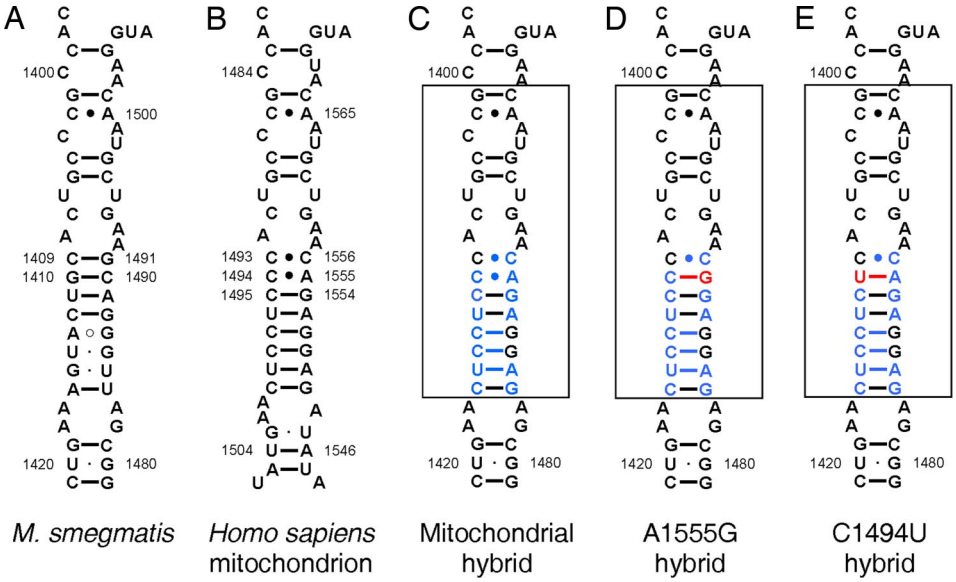


**Fig. 1.** Secondary-structure comparison of decoding-site rRNA sequences in the small ribosomal subunit. (*A*) Decoding region of 16S rRNA helix 44 in wild-type ribosomes of *M. smegmatis*; rRNA nucleotides are numbered according to the bacterial nomenclature, i.e., to homologous *E. coli* 16S rRNA positions. (*B*) Homologous 12S rRNA sequence in human mitochondrial ribosomes; rRNA residues are numbered according to the mitochondrial nomenclature: mtDNA 1494, 1555, and 1556 correspond to *E. coli* 16S rRNA 1410, 1490, and 1491. (*C–E*) Decoding site rRNA of human–bacterial hybrid ribosomes. The transplanted helix is boxed, and nucleotide positions depicted in blue represent residues that are specific for human mitochondrial rRNA. Mutations that are associated with congenital deafness, mtDNA position 1555A to G (*D*), and 1494C to U (*E*) are highlighted in red.

UUC for phenylalanine versus UUA/UUG for leucine. This assay with its artificial mRNA has an intrinsically high error rate (17) and should thus provide a sensitive measure for studying misreading in wild-type compared with mitochondrial hybrid ribosomes. Translation of $AUG(UUU)_{12}$ into oligopeptides composed of the cognate amino acid phenylalanine was comparable in hybrid ribosomes carrying either the mitochondrial wild-type or the A1555G- or C1494U-mutated decoding site (Fig. 2*A* and Table 1). In contrast, the accuracy of mRNA translation was significantly reduced in the hybrid ribosomes carrying a deafness mutation. Misincorporation of the near-cognate amino acid leucine by $AUG(UUU)_{12}$-directed synthesis was increased 4-fold in the C1494U mutant as compared with wild-type mitochondrial and bacterial ribosomes (Fig. 2*A* and Table 1). Hybrid ribosomes carrying the A1555G mutation showed a 7-fold increase in leucine misincorporation relative to the wild-type mitochondrial hybrid. In absolute numbers, the A1555G and C1494U mutant A sites accommodated two leucine tRNAs per 7 and 15 phenylalanine tRNAs, respectively. Thus, although both deafness mutations result in substantial codon misreading, the error frequency is more pronounced in the A1555G mutant.

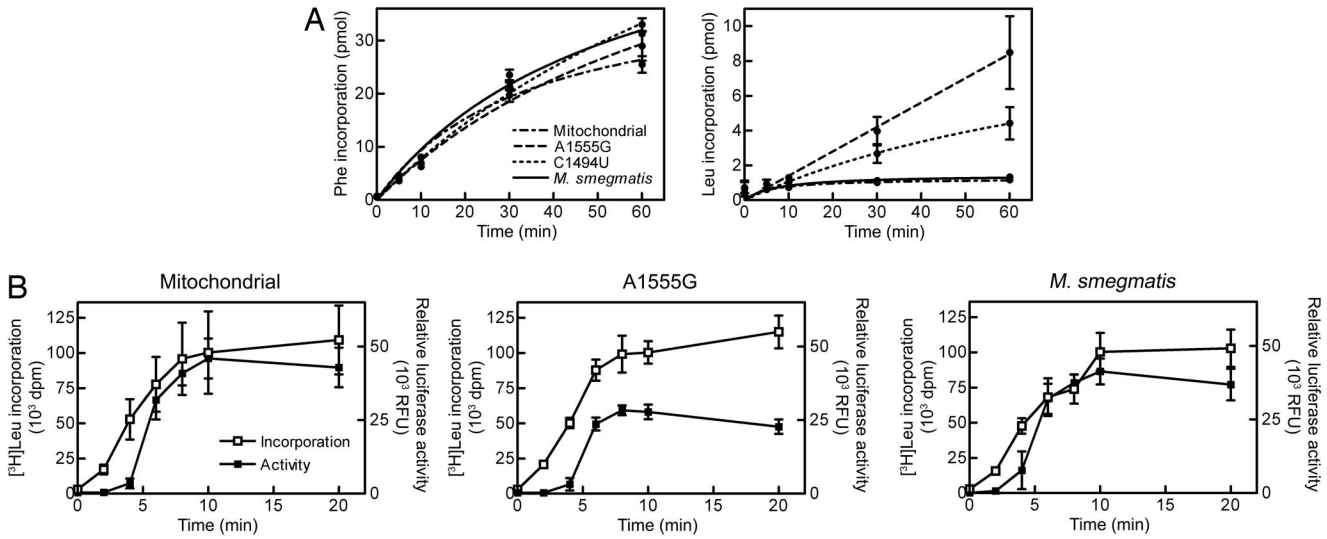


**Fig. 2.** Rate and accuracy of mRNA translation in wild-type and mutant mitochondrial hybrid ribosomes. (*A*) $AUG(UUU)_{12}$ mRNA-directed incorporation of phenylalanine and leucine into oligopeptides synthesized by ribosomes containing either a wild-type mitochondrial, mutant A1555G, or mutant C1494U decoding site (mean ± SD; $n = 3$). *M. smegmatis* wild-type ribosomes are included for comparison. For leucine per phenylalanine ratios, see Table 1. (*B*) Relative bioluminescence in luciferase synthesis assays (■) and total leucine incorporation (□) by mitochondrial hybrid and mutant A1555G ribosomes (mean ± SD; $n = 3$). Bacterial wild-type ribosomes are included for comparison. For luciferase activity per amino acid incorporation ratios, see Table 2.

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**Table 1. Capacity and accuracy of amino acid incorporation**

| | AUG(UUU)$_{12}$-directed amino acid incorporation | | |
|---|---|---|---|
| Decoding site | Phe, pmol | Leu, pmol | Leu/Phe |
| *M. smegmatis* | 31 | 1.3 | 0.04 |
| Mitochondrial | 25 | 1.2 | 0.05 |
| A1555G | 29 | 8.5 | 0.29 |
| C1494U | 33 | 4.4 | 0.13 |
| C1556G | 29 | 0.7 | 0.03 |

Amino acid incorporation is given as the mean amount of [$^{14}$C]phenylalanine and [$^{3}$H]leucine incorporated by 5 pmol of purified ribosomes after 60-min incubation with AUG(UUU)$_{12}$ mRNA as template ($n$ = 3). See Fig. 2*A* and SI Fig. 7 for corresponding time curves and standard deviations.

The codon ambiguity exploited in the AUG(UUU)$_{12}$ assay is located at the third position. Distinction of the third position is relevant for mixed codon boxes, where the third codon base is important for discriminating between the correct cognate or wobble codons, and the near-cognate codons, e.g., UUU/C for Phe versus UUA/G for Leu, CAU/C for His versus CAA/G for Gln, AAU/C for Asn versus AAA/G for Lys, GAU/C for Asp versus GAA/G for Glu (18). To determine ribosome fidelity in translation of a natural mRNA, we used a luciferase-encoding mRNA to analyze synthesis of luciferase based on a coupled transcription–translation assay. The functional enzymatic activity of firefly luciferase [bioluminescence measured as relative fluorescence units (RFU)] was determined and related to the amount of amino acids incorporated (measured as [$^{3}$H]leucine incorporation). Compared with wild-type bacterial and mitochondrial hybrid ribosomes, the mutant A1555G hybrid ribosomes incorporated similar amounts of amino acids in quantitative terms. This observation indicates that the A1555G mutation does not grossly affect ribosome mechanics nor blocks a rate-limiting step in polypeptide synthesis. However, in qualitative terms, the mutant produced ≈50% less functional enzymatic activity per amino acid incorporated (Fig. 2*B* and Table 2). This finding would also be compatible with miscoding, as amino acid misincorporation during the synthesis of luciferase (550 aa in length) would be expected to reduce the enzymatic activity (19), thereby diminishing the level of luminescence without grossly affecting the overall amount of protein synthesized. To distinguish misincorporation from premature termination as cause of decreased luciferase activity, we analyzed the synthesized luciferase by SDS/PAGE and autoradiography. Mutant and wild-type mitochondrial hybrid ribosomes produced the same level of full-length protein (SI Fig. 6).

To study the effect of aminoglycosides on translational fidelity of the deafness alleles, we determined tobramycin-induced misincorporation of leucine in the AUG(UUU)$_{12}$-mRNA translation assay. Fig. 3 shows the dose-dependent exacerbation of translation infidelity in mutant mitochondrial hybrid ribosomes. Wild-type mitochondrial ribosomes are affected only slightly at drug concentrations >50 $\mu$M. The intrinsic infidelity of the A1555G and C1494U mutants, however, is further aggravated by drug concentrations of as little as 2 $\mu$M.

**Table 2. Ribosomal accuracy in luciferase synthesis**

| | Relative luciferase activity per [$^{3}$H]leucine incorporation, RFU/dpm | | |
|---|---|---|---|
| Decoding site | 10 min | 20 min | 30 min |
| *M. smegmatis* | 0.29 | 0.22 | 0.25 |
| Mitochondrial | 0.24 | 0.20 | 0.29 |
| A1555G | 0.11 | 0.12 | 0.13 |

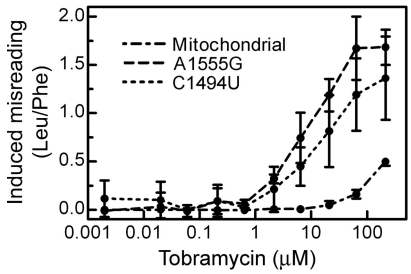


**Fig. 3.** Aminoglycoside-induced exacerbation of translation infidelity. Dose–response curves show tobramycin-induced misincorporation of leucine per phenylalanine by wild-type, A1555G, and C1494U mitochondrial hybrid ribosomes after 60 min incubation with an AUG(UUU)$_{12}$ mRNA template (mean ± SD; $n$ = 3).

Most nucleotides in 16S rRNA helices 18, 34, and 44 that form the small subunit decoding site are universally conserved, although subtle but significant variations have evolved in helix 44. Although the hybrid ribosomes in this study do not carry a complete mitochondrial A site, transplanting the mitochondrial version of helix 44 revealed that, in comparison to the wild-type mitochondrial helix, the disease-associated mutation resulted in significant misreading. How to explain that the deafness mutations C1494U and A1555G result in mistranslation? Kinetic models for tRNA selection suggest that the ribosome discriminates between cognate and noncognate tRNAs by induced fit (20). At the structural level, decoding is linked to conformational changes, in which A1492, A1493, and G530 (*E. coli* numbering) interact intimately with the minor groove of the first two codon-anticodon base pairs, thus allowing for discrimination between cognate and noncognate tRNAs (21) and to initiate transition of the ribosome from an open to a closed form (22). The internal, asymmetric loop containing the bulged-out nucleotides A1492 and A1493 is closed by nucleotides of the lower stem of helix 44 (Fig. 1*A*). Compared with the bacterial A site, where the loop is closed by a Watson–Crick C1409–G1491 interaction, the internal loop in the mitochondrial A site is able to adopt an extended conformation becoming closed further down the helix by a C1495–G1554 base pair (Fig. 1*B*). Both the C1494U and the A1555G mutation replace the noncanonical C1494·A1555 interaction by a Watson–Crick base pairing, thus shortening the extended conformation of the mitochondrial loop. To investigate a possible effect of internal loop closure on mitochondrial decoding accuracy, we introduced a C1493–G1556 base pair to the mitochondrial hybrid (SI Fig. 7*B*). In contrast to a C1494–G1555 interaction, introduction of the C1493–G1556 base pair did not result in miscoding (Table 1 and SI Fig. 7). This finding indicates that closing the bulge does by itself not affect the error rate of mRNA decoding.

The base-pairing pattern of the mitochondrial A1555G or C1494U decoding site resembles that of the bacterial A site carrying a G1491C mutation. In both sites, the C·C opposition at position 1409–1491 (mitochondrial 1493–1556) is followed by a Watson–Crick base pair 1410–1490 (mitochondrial 1494–1555, as depicted in SI Fig. 8*A*). To test whether this more general pattern accounts for the translation phenotype observed in A1555G and C1494U mutant mitochondrial hybrid ribosomes, we determined the translation fidelity of purified G1491C bacterial ribosomes. We found that the bacterial G1491C decoding site shows no significant misreading, but instead exhibits a translation fidelity that compares well with that of wild-type bacterial and wild-type mitochondrial decoding sites (SI Fig. 8*B*). This observation indicates that translation fidelity also depends on base-pair interactions within the lower stem of helix 44, which is part of the entire mitochondrial region transplanted,

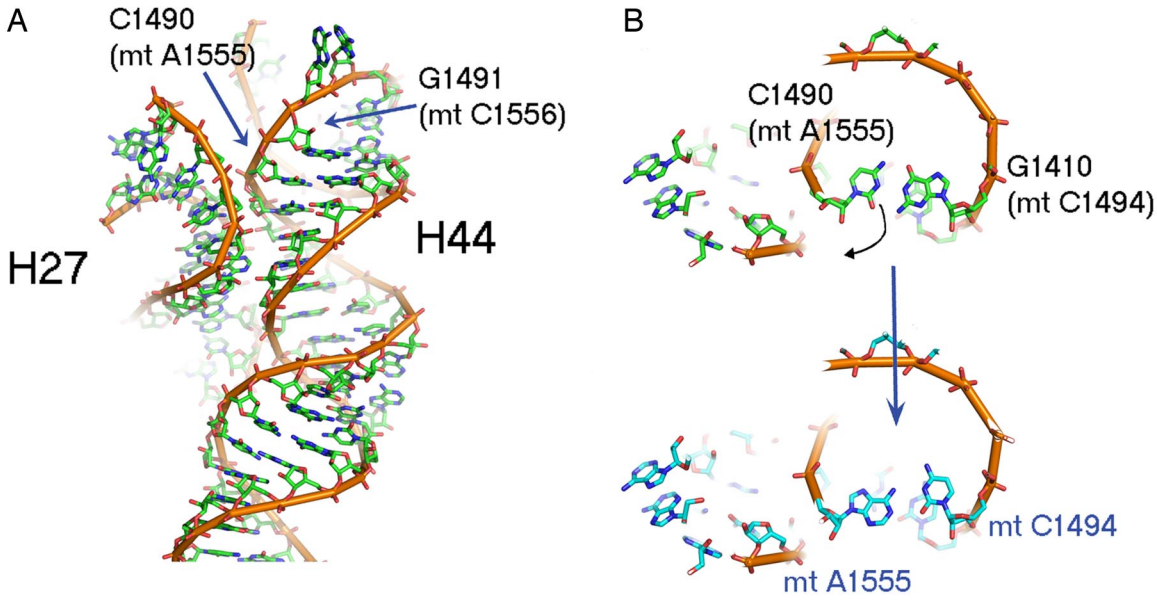


**Fig. 4.** Structural interpretation of mutation A1555G and C1494U. (*A*) The interface of 16S rRNA helices 44 and 27 in the crystal structure of *T. thermophilus* ribosomes (24), showing the bacterial G1410–C1490 base pair, which corresponds to the homologous C1494·A1555 interaction in human mitochondrial 12S rRNA. (*B*) Enlarged view into the vicinity of C1490. The arrow indicates the rotation that C1490 has to undergo (*Upper*) to create a noncanonical C·A interaction present in mitochondrial ribosomes (*Lower*). This rotation increases the interface area between helix 44 and helix 27. Note that owing to the position of nucleotide 1556, the mutation C1556G (*E. coli* G1491) should have no effect on the interface area.

and which is significantly different from its bacterial counterpart (Fig. 1 and SI Fig. 8*A*).

For studying a mechanism for mutation-induced miscoding at the structural level, we made two assumptions: first, the conformation of the decoding region in the native and mutated mitochondrial ribosomes is identical to that of its bacterial counterpart, except for the consequences of known sequence differences; second, the mutations influence their immediate vicinity. For the human cytosolic A site, two theoretical possibilities for a noncanonical C·A base pair have been described based on RNA oligonucleotides: one with a protonated adenine shifted to the minor groove side, the other with a nonprotonated adenine shifted to the major groove side (23). The former is practically superimposable on the *Thermus thermophilus* structure (24, 25) and can explain how codon–anticodon interactions are monitored to ensure translation fidelity. For the latter, no biological correlate has been provided. Modeling the mitochondrial decoding site on the *Thermus* structure revealed that the C1494·A1555 interaction is established by a slight rotation of A1555 toward the outline of helix 44 (Fig. 4). This motion is accompanied by the rotation of C1494 into the helix to create an acceptable donor–acceptor relationship between the adenine and the cytosine. This rotation would increase the surface area between H44 and H27. By introducing a Watson–Crick base pairing, as in C1494–A1555G and C1494U–A1555, nucleotide 1555 will be rotated back into H44, thus reducing the contact between H44 and H27. Destabilizing the interaction between H44 and H27 could make a relative movement between H27 and H44 easier. Such a movement is likely to be part of the induced conformational change required in decoding (22, 26), and lowering the energetic penalty for the change would allow near-cognate tRNAs to be accepted more easily. According to this interpretation, rotation of residue 1555 should be affected by the relative strength of the 1494–1555 interaction: compared with a U–A interaction, a C–G pair would limit the possibility of nucleotide 1555 to escape its displacement imposed by base-pairing with nucleotide 1494; the presence of three as compared with two H bonds provides additional binding energy and makes G–C base pairs intrinsically more stable than A–U pairs. This prediction is in line with the finding that the A1555G mutation results in a more pronounced misreading than the C1494U mutation.

Simple single-cell prokaryotic organisms are apparently able to tolerate significant misreading (27, 28), as also evidenced by the viability of the A1555G and C1494U hybrid mutants. However, the situation in complex multicellular eukaryotes and in highly specialized tissues is notably different. Misfolded proteins have been implicated in a variety of diseases (29), in particular involving cell types that have limited ability to regenerate by cell division. At least two mechanisms may account for the disease phenotype conferred by mistranslation: dysfunctional proteins and misfolded protein response (30). The 13 proteins encoded by human mtDNA and translated by mitochondrial ribosomes are subunits of the respiratory chain and oxidative phosphorylation pathway at the inner mitochondrial membrane. Lymphoblastoid cell lines with mutations in mitochondrial 12S rRNA have been reported to show decreased rates of oxygen consumption (9, 10). In cell lines of patients with the A1555G mutation no alterations of mitochondrial translation products at the level of electrophoretic mobility were found (31). This observation is compatible with our luciferase data in the mitochondrial hybrid ribosomes, which demonstrate that mutation-mediated misreading manifests primarily at the level of functional activity of the translated protein by missense rather than by nonsense decoding (SI Fig. 6). Increased mistranslation of mitochondrial genes might also account for a misfolded protein response to result in the cochlear alterations observed in symptomatic and asymptomatic carriers of the A1555G mutation (11).

Identification of misreading as key mechanism in pathogenesis also allows us to integrate the role of the nuclear background in disease manifestation and address the association of the A1555G and C1494U mutations with aminoglycoside-induced deafness. The nuclear-encoded proteins thus far linked to the expression of the deafness phenotype are involved in mitochondrial tRNA or rRNA modifications (32–34). On the basis of a misreading-prone ribosome, any change in translational efficiency, regard-

less of its specific mechanism of action, would further impair mitoribosome function, to aggravate translational dysfunction beyond the threshold required to result in the phenotypic expression of severe hearing loss that is associated with mutations A1555G and C1494U. Likewise, the misreading phenotype of the A1555G and C1494U mutant mitochondrial ribosomes is significantly exacerbated by aminoglycoside antibiotics.

Although the basis for the tissue specificity of disease expression is unclear, the biochemical phenotype of the mitochondrial deafness alleles, as revealed by the study of hybrid ribosomes, is identical to that of aminoglycoside action, i.e., increased mistranslation. Together with the dose-related, irreversible ototoxicity of aminoglycosides, which presumably at least in part reflects drug-mediated dysfunction of the mitochondrial ribosome (8), we conclude that the findings we observe are significant in understanding the mechanisms of mitochondrial deafness. We can presently not exclude that, in addition to mistranslation, other aspects of ribosome function may result in pathogenic properties of the mutant. Although additional effects may be involved, our results provide a mechanistic link between mtDNA deafness mutations and pathogenesis. The experimental model developed here may help in the identification of prokaryotic homologues of nuclear genes modifying phenotypic expression of the disease and in the design of RNA-specific agonists for treatment. As a further outcome of our work, we note that identification of error-prone protein synthesis in mitochondria with an A1555G mutation also allows testing of the long-standing hypothesis of mitochondrial dysfunction and aging (35).

## Materials and Methods

**Construction of Mutant Strains with Hybrid Ribosomes.** The recently described strain *M. smegmatis* Δ*rrnB* (16) was used for all genetic manipulations. Site-directed mutagenesis of its single rRNA operon was performed by PCR mutagenesis with hybrid rDNA oligonucleotides comprising the wild-type or mutant mitochondrial helix 44 decoding site sequence. The resulting hybrid gene fragment was cloned into an integration-proficient plasmid used to transform *M. smegmatis* Δ*rrnB*. Transformants were selected on LB agar plates containing 20 μg/ml paromomycin for gene replacement by homologous recombination. After several attempts to introduce various parts of the mitochondrial H44 into bacterial ribosomes by site-directed mutagenesis, we were finally able to generate recombinant *M. smegmatis* cells where the central 34-nt part of the bacterial H44 had been replaced by its mitochondrial counterpart. Successful replacement of the bacterial decoding site sequence with the mitochondrial sequence was controlled by sequence analysis of the chromosomal *rrnA* locus. Transplanting the mitochondrial decoding sites into the bacterial ribosome affected the generation times of the resulting *M. smegmatis* mutants slightly (mitochondrial hybrid 5.1 h; C1494U 4.9 h; A1555G 6.0 h; as compared with *M. smegmatis* ΔrrnB 3.7 h).

**Isolation and Purification of Ribosomes.** Ribosomes were isolated from bacterial cell pellets as described (36). For further fractionation, isolated ribosomes were resuspended in overlay buffer [20 mM Tris·HCl (pH 7.4), 60 mM $NH_4Cl$, 5.25 mM $MgCl_2$, 0.25 mM EDTA, 3 mM 2-mercaptoethanol, and 5% sucrose] loaded on a sucrose gradient (10–40% sucrose in overlay buffer) and centrifuged in a Beckman Ti 15 rotor at 28,000 rpm for 18 h. Gradient fractions were collected by unloading the zonal rotor with 50% sucrose in overlay buffer. The 70S ribosome-enriched fractions were pooled according to the absorption profile at 260 nm, and applied to centrifugation at 180,000 × *g* for 20 h. The final ribosome pellets were resuspended in buffer A [50 mM Tris·HCl (pH 7.5), 70 mM $NH_4Cl$, 30 mM KCl, and 7 mM $MgCl_2$], incubated for 30 min at 4°C, dispensed into aliquots, and stored at −80°C after shock freezing in liquid nitrogen. 70S ribosome concentrations were determined by absorption measurements on the basis of 23 pmol per $A_{260}$ unit. Integrity and functional activity of purified 70S ribosomes was determined by analytical ultracentrifugation and by assessing their capacity to form initiation complexes, as described (36).

**Cell-Free AUG(UUU)$_{12}$ Translation Assays.** Cell-free translation reactions in buffer A (pH 7.5) were prepared on ice and contained *M. smegmatis* $tRNA^{bulk}$ (0.5 mg/ml), amino acids mixture (30 μM each) lacking phenylalanine and/or leucine, 10% (vol/vol) S100 extract, energy mix (1 mM DTT, 1 mM GTP, 4 mM ATP, and 5 mM phospho*enol*pyruvate), pyruvate kinase (0.1 mg/ml), and polyamines (2 mM spermidine and 8 mM putrescine). The reaction mixture was preincubated with 30 μM [$^{14}$C]phenylalanine (110 mCi/mmol) and/or 30 μM [$^{3}$H]leucine (500 mCi/mmol) at 37°C for 15 min. The translation reaction was started by addition of ribosomes to a final concentration of 0.25 μM and AUG(UUU)$_{12}$-mRNA [5′-GCGGCAAGGAGGUAAAUAAUG(UUU)$_{12}$UAAGCAGG-3′, obtained from Dharmacon] to 1 μM; in experiments studying tobramycin-induced misreading, the drug was added simultaneously. After incubation at 37°C for the times indicated, the reaction was stopped by addition of KOH to 0.5 M and subsequent hydrolysis at 37°C for 30 min. Synthesized polypeptides were precipitated with 200 μl of 5% trichloroacetic acid (TCA) for 10 min on ice and bound to filters. Filter-bound polypeptides were washed with cold 30% 2-propanol, dissolved in 10 ml of scintillation mixture, and quantified.

**Cell-Free Luciferase Translation Assays.** Purified 70S hybrid ribosomes were used in a coupled transcription-translation reaction of firefly luciferase (plasmid pBESTluc; Promega). A typical reaction (30 μl volume) contained 0.25 μM 70S ribosomes, 600 ng DNA, 40% (vol/vol) of *M. smegmatis* S100 extract, 100 μM amino acid mixture minus leucine, 1.7 μM [$^{3}$H]leucine (59 Ci/mmol), and RNasin (40 units; Promega). rNTPs, tRNAs, and energy were supplied by the addition of commercial S30 Premix Without Amino Acids (Promega). The reaction mixture was incubated at 37°C and stopped on ice. Fifteen microliters of the reaction volume was added to 100 μl of luciferase assay substrate (Promega), and the bioluminescence was measured in a luminometer (Bio-Tek Instruments; FL ×800). In the remaining 15 μl of reaction volume, polypeptides were hydrolyzed, precipitated, applied to a GF/A filter (Whatman), and washed three times with 3 ml of ice-cold 5% TCA. Filters were air-dried for 10 min, and [$^{3}$H]leucine incorporation was quantified in 10 ml of scintillation mixture.

**Structural Modeling.** Modeling the mitochondrial decoding site on the *Thermus thermophilus* structure (24) was done manually, according to the following procedure: (*i*) substituting the bacterial residues G1410 with C and C1490 with A according to the homologous positions in mitochondrial ribosomes (C1494·A1555), using the backbone conformation as a guide; (*ii*) fitting A1555 into the helix so that a noncanonical C·A base pair could be formed; and (*iii*) combining attempts to obtain a relevant H bond with estimating (by actual measurements) the deviation of the new structure from a perfect helix and searching for structural elements that can be exploited for the extra stabilization needed for the slightly shifted-rotated region of the helix. In addition to the slight rotation, a small shift was included in the modeling; the latter, however, is of negligible magnitude at the resolution level of the structure.

**ACKNOWLEDGMENTS.** We thank Raz Zarivach, Anat Bashan, and Ada Yonath for valuable advice on structural interpretations, Marina Rodnina (Universität Witten/Herdecke, Witten, Germany) for generously providing [$^{3}$H]fMet-$tRNA^{fMet}$; and Tanja Janušić for expert technical assistance. This work was supported by grants from the Swiss National Science Foundation (to E.C.B.) and the Bonizzi-Theler-Stiftung (to S.N.H.).

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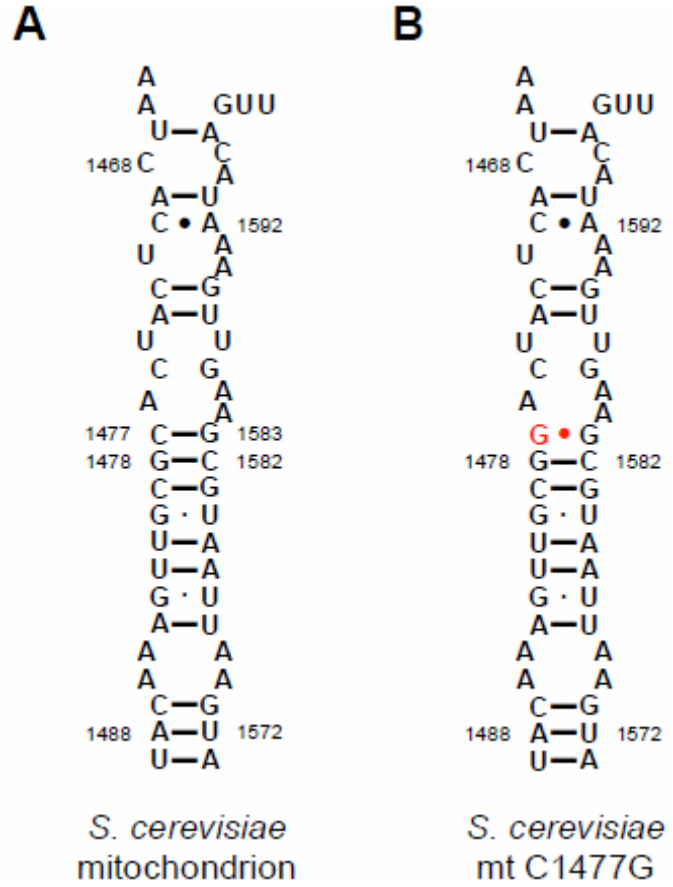
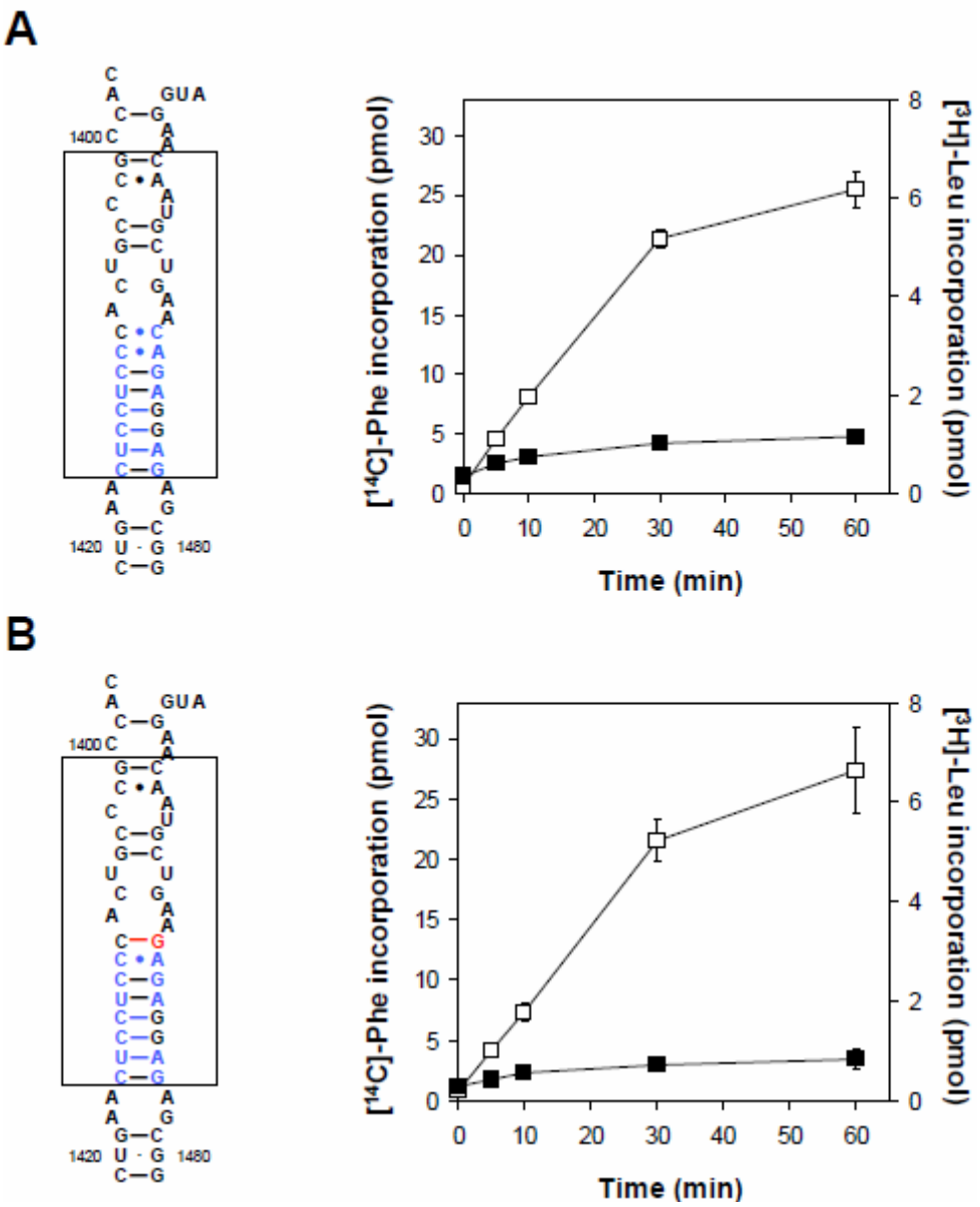
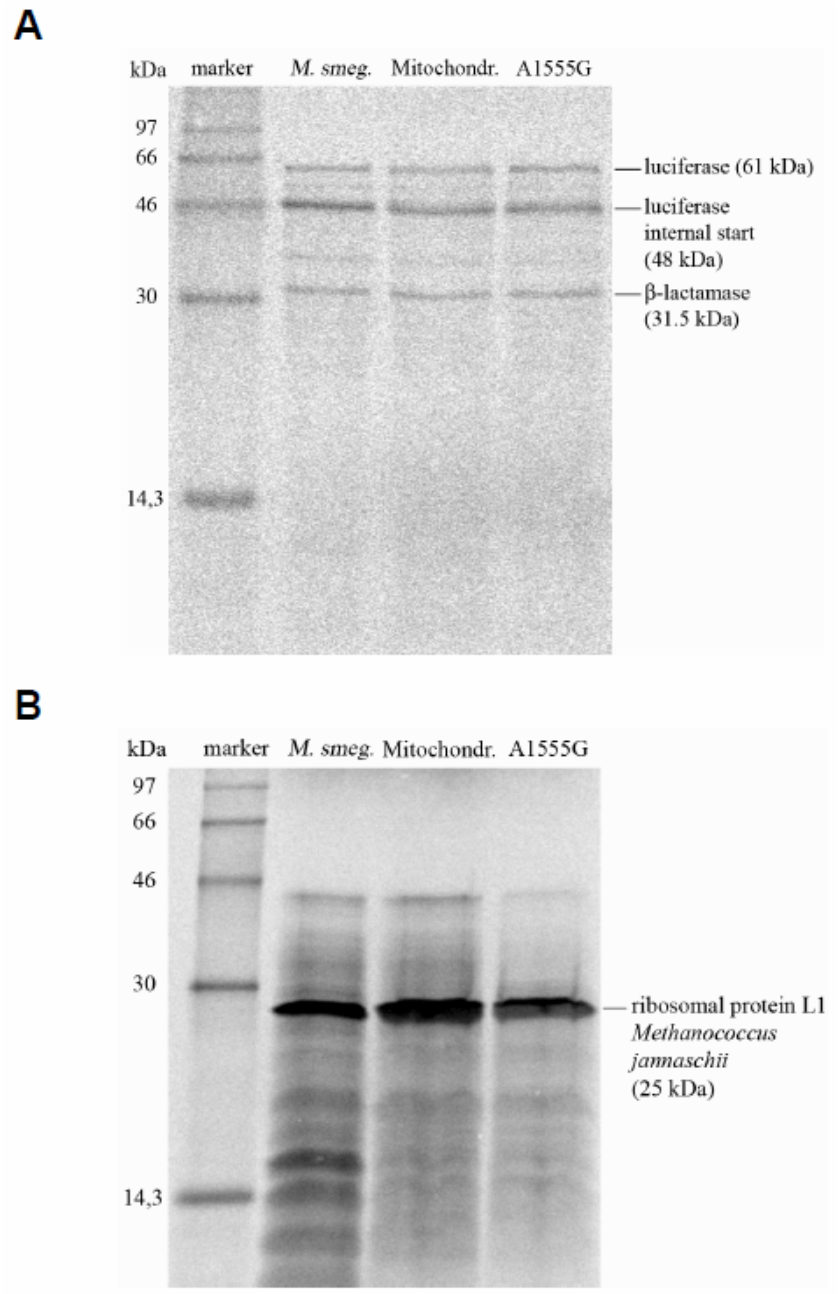
**SUPPORTING INFORMATION**



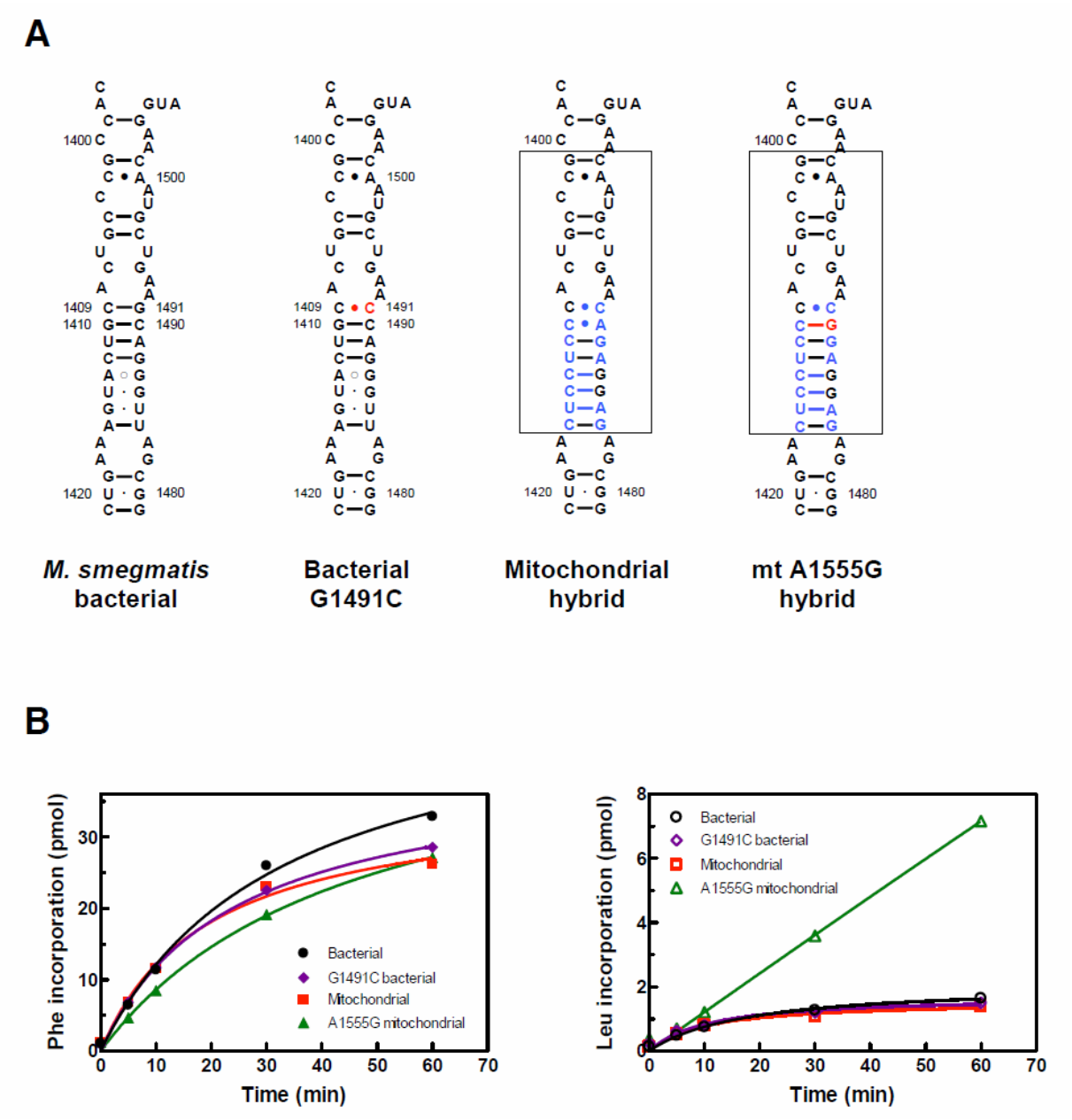

**SI Figure 5:** Secondary structure of the rRNA decoding site in yeast mitochondria. (*A*) The ribosomal decoding site of *Saccharomyces cerevisiae* mitochondrial ribosomes; rRNA residues are numbered according to the position in yeast mtDNA. (*B*) Mitochondrial mutant decoding site carrying a cytosine to guanine mutation at position 1477 (corresponding to *E. coli* position 1409). Note that yeast mt rRNA position 1477 is not homologous to human mt rRNA position 1494 but to position 1493 (compare to Figure 1 in the article). In addition, the lower stem of the yeast mitochondrial rRNA helix resembles its bacterial homologue helix 44 rather than its human mitochondrial counterpart: e.g. a Watson-Crick interaction at yeast mt rRNA positions C1477–G1583 (bacterial C1409–G1491) and G1478–C1582 (bacterial G1410–C1490), the corresponding human mt rRNA positions C1493●C1556 and C1494●A1555 are no Watson-Crick interactions.




**SI Figure 6:** Capacity and accuracy of translation in mitochondrial hybrid and C1556G ribosomes. AUG(UUU)$_{12}$ mRNA-directed incorporation of [$^{14}$C]-phenylalanine (□) and [$^{3}$H]-leucine (■) in a cell-free translation assay (mean ± s. d.; *n* = 3). (*A*) Bacterial hybrid ribosomes with a wild-type mitochondrial decoding site. (*B*) Mutant mitochondrial hybrid ribosomes with a cytosine to guanine alteration at position 1491 (mtDNA 1556), which creates a canonical C–G base pair interaction. The ratio of leucine per phenylalanine incorporation is given in Table 1.




**SI Figure 7:** To distinguish misincorporation from premature termination we analyzed [$^{35}$S]-methionine-labeled proteins synthesized in cell-free translation assays by purified bacterial and mitochondrial hybrid ribosomes using SDS-PAGE (12%) fluorography. Mutant A1555G and wild-type mitochondrial hybrid ribosomes produced the same level of full-length proteins, as assessed by translation of luciferase mRNA in a coupled transcription-translation reaction and by translation of L1 mRNA in T7-mRNA driven translation. (*A*) Proteins translated during luciferase coupled transcription-translation showing full length luciferase (61 kDa), truncated luciferase transcribed from an internal start codon (48 KDa), and β-lactamase (31.5 kDa). (*B*) T7-mRNA-driven synthesis of *Methanococcus jannaschii* ribosomal protein L1 (25 kDa).




**SI Figure 8:** Comparison of the mitochondrial A1555G mutant with the bacterial G1491C mutant. (*A*) Structural comparison of the base-pairing pattern in the ribosomal A site. Both the A1555G and the G1491C mutant are characterized by a C•C opposition at position 1409-1491 (mitochondrial 1493-1556, respectively) and an adjacent C-G Watson-Crick pair. (*B*) Translation phenotypes of wild-type and mutant bacterial and mitochondrial decoding sites. The amount of [$^{14}$C]-phenylalanine and [$^{3}$H]-leucine incorporated in a AUG(UUU)$_{12}$ mRNA-driven translation assay is presented.

# Genetic analysis of interactions with eukaryotic rRNA identify the mitoribosome as target in aminoglycoside ototoxicity

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Communicated by Ada Yonath, Weizmann Institute of Science, Rehovot, Israel, November 7, 2008 (received for review July 28, 2008)

**Aminoglycoside ototoxicity has been related to a surprisingly large number of cellular structures and metabolic pathways. The finding that patients with mutations in mitochondrial rRNA are hypersusceptible to aminoglycoside-induced hearing loss has indicated a possible role for mitochondrial protein synthesis. To study the molecular interaction of aminoglycosides with eukaryotic ribosomes, we made use of the observation that the drug binding site is a distinct domain defined by the small subunit rRNA, and investigated drug susceptibility of bacterial hybrid ribosomes carrying various alleles of the eukaryotic decoding site. Compared to hybrid ribosomes with the A site of human cytosolic ribosomes, susceptibility of mitochondrial hybrid ribosomes to various aminoglycosides correlated with the relative cochleotoxicity of these drugs. Sequence alterations that correspond to the mitochondrial deafness mutations A1555G and C1494T increased drug-binding and rendered the ribosomal decoding site hypersusceptible to aminoglycoside-induced mistranslation and inhibition of protein synthesis. Our results provide experimental support for aminoglycoside-induced dysfunction of the mitochondrial ribosome. We propose a pathogenic mechanism in which interference of aminoglycosides with mitochondrial protein synthesis exacerbates the drugs' cochlear toxicity, playing a key role in sporadic dose-dependent and genetically inherited, aminoglycoside-induced deafness.**

decoding | mitochondria | ribosomes | toxicity | translation

Low cost and high efficacy make aminoglycosides a common choice for treatment of serious infections caused by gram-negative bacilli, including endocarditis, sepsis, pneumonia, pyelonephritis, and multidrug-resistant tuberculosis (1). Unfortunately, aminoglycosides are both nephrotoxic and ototoxic. Although renal impairment is in general mild and reversible, ototoxicity results from drug-induced apoptosis of cochlear and vestibular hair cells and is irreversible (2, 3). Ototoxicity of aminoglycoside antibiotics occurs both in a dose-dependent and in an inherited idiosyncratic fashion. Despite attempts to limit drug doses and to monitor blood levels carefully, measurable signs of hearing loss are found in 20% of patients receiving aminoglycosides (2). Familial cases of aminoglycoside-induced deafness are maternally transmitted and linked to mutations in mitochondrial DNA (mtDNA) (4–6).

The mechanisms by which aminoglycoside antibiotics exert their toxic effects are controversial. A surprisingly large and diverse number of effects have been associated with aminoglycosides. Aminoglycosides have been reported to affect DNA, RNA, and protein synthesis; energy metabolism and ion transport; and synthesis or degradation of prostaglandins, gangliosides, mucopolysaccharides and lipids (2). In addition, it has been hypothesized that aminoglycosides may form cochleotoxic metabolites. Antioxidants apparently attenuate aminoglycoside-induced hearing loss, pointing to a role of the mitochondrion, an organelle involved in oxidation, as a target of ototoxic drugs (7, 8). Genetic analyses of individuals hypersensitive to aminoglycosides have identified mutations in mitochondrial rRNA. Transition mutations in the mitochondrial small ribosomal RNA gene, namely A1555G and, less frequently C1494T, have been identified as primary genetic traits in aminoglycoside-induced deafness (4, 6, 9). A1555G and C1494T both map to the aminoacyl-tRNA acceptor site (A site) of the small ribosomal subunit. The bacterial A-site rRNA is target for aminoglycoside antibiotics, which exert their antibacterial effect at the level of the prokaryotic ribosome (10–13). Aminoglycosides affect protein synthesis by inducing codon misreading and by inhibiting translocation of the tRNA-mRNA complex (14, 15). The basis for aminoglycoside selectivity is presumably their preferential binding to the bacterial as opposed to eukaryotic ribosomes (13, 16, 17).

The high copy number of mtDNA in mitochondria and the vast number of mitochondria in a single cell have frustrated any attempt of genetic manipulation of mitochondrial rRNA in lower and higher eukaryotes. Model oligonucleotides designed to mimic the drug-binding site have been used to investigate various aspects of aminoglycoside-ribosome interaction (18–22). However, conclusions derived from the study of model A-site oligonucleotides are compromised by several findings: (*i*) in contrast to drug susceptibility of complete ribosomes, binding affinities of aminoglycosides to prokaryotic decoding region constructs are not very sensitive to mutations within the RNA-binding region (23); (*ii*) *in vivo* drug susceptibilities of mutant ribosomes and *in vitro* binding affinities using variants of model A-site oligonucleotides may or may not correlate (24–26); (*iii*) the exquisite specificity of aminoglycosides for the prokaryotic as opposed to the eukaryotic cytosolic ribosome contrasts with the observation that these drugs bind to eukaryotic decoding-site constructs with approximately the same affinity as found for their prokaryotic counterpart (23, 24); and (*iv*) while there is evidence that mitochondrial ribosomes are susceptible to aminoglycosides (13, 27), oligonucleotides mimicking the mitochondrial A site do not bind aminoglycosides to any significant extent (24, 28).

Using gene-shuffling experiments, we have previously replaced the A-site residues of helix 44 (H44) in bacterial 16S rRNA with various eukaryotic homologues, demonstrating that the A-site rRNA behaves as an autonomous domain, which can be exchanged between different species for study of function (29,

Author contributions: E.C.B. designed research; S.N.H., S.A., S.K.K., C.M.B., and D.S. performed research; S.N.H., S.A., S.K.K., C.M.B., D.S., and E.C.B. analyzed data; and S.N.H. and E.C.B. wrote the paper.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0811258106/DCSupplemental.

**Table 1. Minimal inhibitory concentrations ($\mu$g/ml) of aminoglycoside antibiotics**

| | A-site rRNA | | | |
|---|---|---|---|---|
| Aminoglycoside | Bacterial[a] | Mitochondrial (mt) hybrid | mt A1555G hybrid | mt C1494T hybrid |
| Neomycin B | 0.5 | 16–32 | 8 | 8 |
| Paromomycin | 1 | > 1,024 | 256–512 | 256–512 |
| Kanamycin A | 1 | 256–512 | 16–32 | 16 |
| Tobramycin | 1 | 128 | 16 | 16 |
| Amikacin | 0.5 | 32–64 | 2–4 | 2–4 |
| Gentamicin | 1 | 64–128 | 16–32 | 16–32 |
| Netilmicin | 2 | 512–1024 | 64 | 64–128 |

[a]*M. smegmatis* wild-type rRNA

30). Replacement of a 34-nucleotide portion of bacterial 16S-rRNA helix 44 with its human homologues resulted in rRNA-decoding sites virtually identical to that in cytosolic and mitochondrial ribosomes. Here we used hybrid bacterial ribosomes carrying distinct alleles of the mitochondrial decoding site to study aminoglycoside susceptibility of wild-type and mutant mitochondrial rRNA.

## Results and Discussion

The *in vivo* activity of various 2-deoxystreptamine antibiotics against isogenic *Mycobacterium smegmatis* strains carrying mitochondrial-bacterial hybrid ribosomes was tested in minimal inhibitory concentration (MIC) assays, which determine growth inhibition at the whole-cell level. Compared to bacterial ribosomes, which were found to be unanimously susceptible to all aminoglycosides tested, the hybrid ribosomes with a wild-type mitochondrial H44 revealed a heterogeneous drug susceptibility pattern, with MIC values ranging from 32 to 1,024 $\mu$g/ml (Table 1). The ratio of MIC mitochondrial hybrid to MIC wild-type *M. smegmatis* varied from 64-fold (gentamicin, amikacin) to 256-fold (netilmicin, kanamycin), providing a relative measure of the drug-target selectivity of different 2-deoxystreptamine antibiotics. We next investigated recombinants where the bacterial H44 has been replaced by mitochondrial deafness alleles corresponding to mtDNA mutations A1555G and C1494T. The resulting mutant mitochondrial hybrid ribosomes differ from the wild-type mitochondrial hybrid only in 16S rRNA residues 1490 and 1410; compare Fig. 1*C* to Fig. 1 *D* and *E* (bacterial 16S rRNA residues are numbered according to *Escherichia coli* nomenclature). The presence of the A1555G or the C1494T mutation increased drug susceptibility of cells carrying the mitochondrial hybrid ribosomes by 4- to 16-fold (see Table 1).

For a more detailed study of the mt A1555G and C1494T alleles, we studied purified hybrid ribosomes in cell-free translation reactions. We first used an AUG(UUU)$_{12}$-mRNA template, as this message allows determination of drug-induced inhibition of polypeptide synthesis and amino acid misincorporation. Dose-response curves of aminoglycoside-induced inhibition of phenylalanine incorporation were analyzed to define the IC$_{50}$ values of the individual 2-deoxystreptamines. Both the A1555G and the C1494T genotypes were more susceptible to aminoglycoside antibiotics than the wild-type mitochondrial decoding site, as indicated by the finding that significantly less drug concentrations were required to inhibit AUG(UUU)$_{12}$ mRNA-driven polyPhe synthesis [see Fig. 2*A*, Table 2, and supporting information (SI) Fig. S1].

Aminoglycosides are known to affect the translational fidelity of ribosomes by inducing misreading of the genetic code (14). For study of aminoglycoside-induced mistranslation we used the AUG(UUU)$_{12}$-driven polypeptide synthesis assay to determine

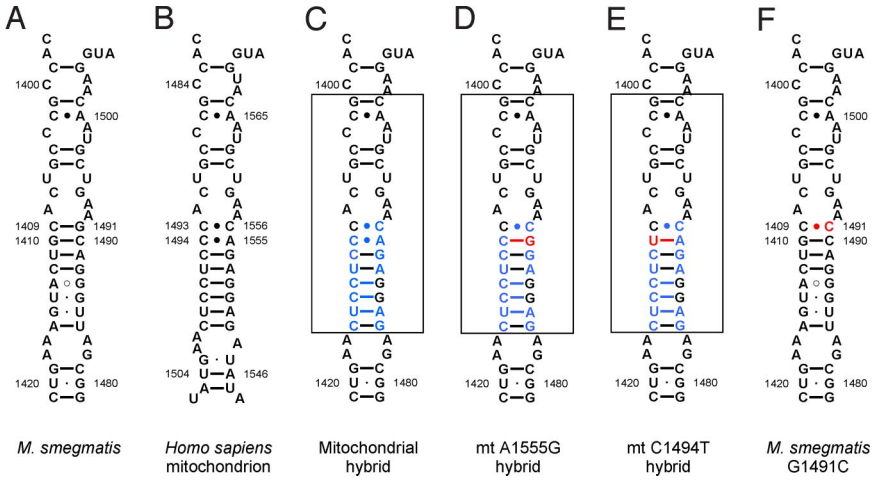


**Fig. 1.** Secondary structure of rRNA helix 44 in the ribosomal decoding site. (*A*) Decoding site of *M. smegmatis* wild-type ribosomes; rRNA nucleotides are numbered according to the bacterial nomenclature (i.e., homologous *E. coli* 16S rRNA positions). (*B*) Decoding site of human mitochondrial ribosomes; rRNA residues are numbered according to the mitochondrial nomenclature. (*C–E*) Mitochondrial decoding sites within human-bacterial hybrid ribosomes: wild type sequence (*C*) and deafness-associated alterations adenine to guanine at position 1490 (corresponding to mitochondrial mutation A1555G) (*D*); cytosine to uracil mutation at position 1410 (corresponding to mitochondrial mutation C1494T) (*E*). Nucleotide positions depicted in blue represent residues that are specific for human rRNA; nucleotide positions in red highlight the pathogenic mutations; the transplanted helix is boxed. (*F*) Decoding site of the *M. smegmatis* G1491C mutant.

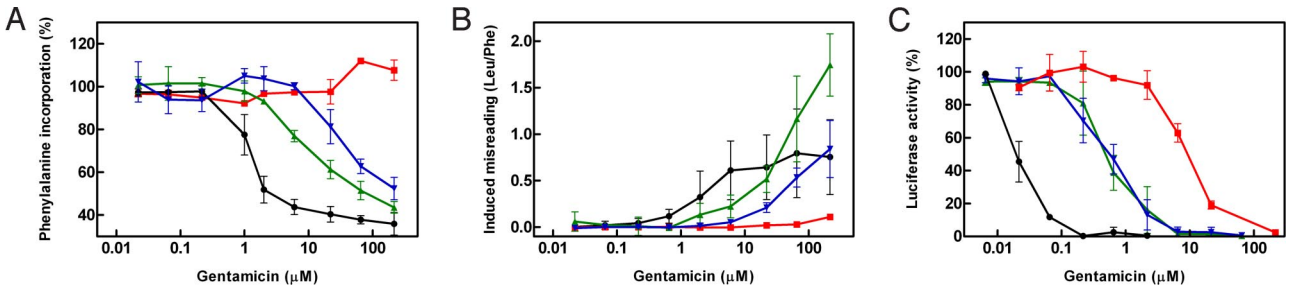


**Fig. 2.** Aminoglycoside susceptibility of mutant and wild-type mitochondrial hybrid ribosomes. Dose-response curves of wild-type mitochondrial (red squares), mutant A1555G (green triangles), and mutant C1494T (blue inverted triangles) hybrid ribosomes; bacterial ribosomes (black circles) are included for comparison. (*A*) Gentamicin-induced inhibition of [$^{14}$C]-phenylalanine incorporation ($n \geq 3$; ± SD.). The 100% value corresponds to 25 to 30 pmol Phe incorporation. Corresponding $IC_{50}$ values of gentamicin and selected aminoglycoside antibiotics are presented in Table 2. (*B*) Gentamicin-induced increase in misincorporation of the near-cognate [$^{3}$H]-leucine relative to the drug-free control ($n \geq 3$; ± SD.). (*C*) Gentamicin-induced inhibition of luciferase synthesis relative to the drug-free control ($n \geq 3$; ± SD.). Corresponding $IC_{50}$ values for gentamicin and selected aminoglycoside antibiotics are presented in Table 3.

the relative amount of near-cognate leucine incorporation compared to incorporation of the cognate amino acid phenylalanine in the presence of various concentrations of gentamicin. Relative incorporation of [$^{3}$H]-labeled leucine versus [$^{14}$C]-labeled phenylalanine was determined and plotted against gentamicin concentration. Introduction of the A1555G and C1494T alteration rendered the mitochondrial hybrid ribosomes highly susceptible to aminoglycoside-induced misreading (Fig. 2*B*; see also Fig. S2). In quantitative terms, the amount of gentamicin-induced misreading (calculated as leucine per phenylalanine incorporation) in A1555G and C1494T mutant hybrid ribosomes was up to 1.75 leucine per phenylalanine, as compared to a maximum of 0.2 leucine per phenylalanine for hybrid ribosomes with a wild-type mitochondrial decoding site.

To study the effect of aminoglycoside antibiotics on translation of a more natural mRNA template, we tested wild-type and mutant mitochondrial hybrid ribosomes in a cell-free luciferase synthesis assay. As depicted in Fig. 2*C*, Table 3, and Fig. S3, the allele- and drug-specific inhibition of luciferase synthesis essentially correlated with the results of the MIC and AUG(UUU)$_{12}$ assays. Drug-mediated inhibition of luciferase synthesis was significantly increased in A1555G and C1494T mutant ribosomes.

The basis for the selectivity of aminoglycosides is presumably their preferential binding to bacterial as opposed to eukaryotic ribosomes (10, 13, 16, 17). In particular, 16S rRNA nucleotides 1408, 1409, and 1491 of helix 44 have been shown to be critical for drug-binding by forming direct contacts with ring I of the 2-deoxystreptamines (12, 13, 16, 17, 26, 31–38) (see Fig. S4 for the chemical structures of aminoglycosides used in this study). In the absence of X-ray structures for aminoglycosides complexed to the mitochondrial ribosome, we can rationalize our findings on data invoked from the study of bacterial ribosome-drug complexes (12, 39). The rRNA secondary structure of the drug binding site in mitochondrial A1555G and C1494T mutant ribosomes resembles that of bacterial ribosomes with a G1491C alteration in that the C1409-C1491 opposition is accompanied by a 1410–1490 Watson–Crick pair (see Fig. 1). The bacterial G1491C ribosome shows a drug-susceptibility phenotype that is virtually superimposable on that found for the mitochondrial deafness alleles (see Table S1 for comparison). To determine whether the affinity of aminoglycosides to mutant mitochondrial decoding sites corresponds to that of the bacterial G1491C decoding site, we probed gentamicin binding by chemical footprinting experiments. Bacterial wild-type ribosomes showed drug-mediated protection from dimethyl sulfate (DMS) modification at G1405 (N-7), which is in good agreement with previous reports on aminoglycoside protection in bacterial 16S rRNA (34). Wild-type mitochondrial hybrid ribosomes showed little protection, while mutant mitochondrial A1555G and C1494T hybrid ribosomes showed a concentration-dependent protection of G1405 that resembles the dose-response curve observed with bacterial G1491C ribosomes (Fig. 3). Thus, binding of aminoglycosides to ribosomes with an adenine at 16S rRNA position 1408, appears to be mainly determined by the structural geometry of base pairs 1409–1491 and 1410–1490.

When studying drug-induced miscoding, we found that the decoding accuracy of bacterial G1491C ribosomes is barely affected by aminoglycoside antibiotics. In absolute terms and in contrast to the mitochondrial A1555G and C1494T deafness mutants, the bacterial G1491C ribosomes showed little drug-

**Table 2. Aminoglycoside-induced inhibition of AUG(UUU)$_{12}$-driven phenylalanine incorporation ($IC_{50}$, μM)**

| | A-site rRNA | | | |
|---|---|---|---|---|
| Aminoglycoside | Bacterial[a] | Mitochondrial (mt) hybrid | mt A1555G hybrid | mt C1494T hybrid |
| Neomycin B | 0.2 ± 0.0 | 209 ± 61 | 3.1 ± 0.3 | 3.7 ± 0.7 |
| Paromomycin | 0.7 ± 0.1 | > 500 | 124 ± 35 | 116 ± 20 |
| Kanamycin A | 1.2 ± 0.2 | > 500 | 20 ± 2 | 95 ± 29 |
| Tobramycin | 0.7 ± 0.1 | > 500 | 15 ± 3 | 70 ± 23 |
| Amikacin | 0.8 ± 0.1 | > 500 | 7.1 ± 0.8 | 13 ± 3 |
| Gentamicin | 1.3 ± 0.2 | > 500 | 13 ± 2 | 62 ± 16 |
| Netilmicin | 1.2 ± 0.3 | > 500 | 71 ± 10 | 331 ± 84 |

$IC_{50}$ values represent the drug concentrations in μM that are required to inhibit AUG(UUU)$_{12}$-driven phenylalanine incorporation to half-maximal extent. A representative graph showing phenylalanine incorporation plotted against gentamicin concentration is shown in Fig. 2*A*.

[a]*M. smegmatis* wild-type rRNA

**Table 3. Aminoglycoside-induced inhibition of luciferase synthesis ($IC_{50}$, $\mu$M)**

| | A-site rRNA | | | |
|---|---|---|---|---|
| Aminoglycoside | Bacterial[a] | Mitochondrial (mt) hybrid | mt A1555G hybrid | mt C1494T hybrid |
| Neamine | 1.4 | 131 | 32 | 35 |
| Neomycin B | 0.04 | 0.7 | 0.5 | 0.4 |
| Paromomycin | 0.03 | 33 | 2.4 | 3.1 |
| Kanamycin A | 0.05 | 15.7 | 1.2 | 1.1 |
| Tobramycin | 0.02 | 7.8 | 0.8 | 0.9 |
| Amikacin | 0.02 | 7.0 | 0.4 | 0.6 |
| Gentamicin | 0.03 | 5.7 | 0.6 | 0.7 |
| Netilmicin | 0.05 | 17.6 | 0.8 | 2.6 |

$IC_{50}$ values represent the drug concentrations in $\mu$M that are required to inhibit synthesis of functional firefly luciferase to 50%. Relative luciferase activity plotted against aminoglycoside concentration is shown in Fig. 2*C*.
[a]*M. smegmatis* wild-type rRNA

induced misreading (see Fig. S2). The bacterial G1491C mutant and the hybrid deafness ribosomes differ primarily in 16S rRNA residues 1413 to 1415 and 1485 to 1487, which form the lower stem of helix 44 (see Fig. 1). At the structural level, helix 44 interacts with helix 27. By modeling, nucleotide alterations in the lower stem of H44 have been suggested to affect this interaction and the relative movement between these two helices as part of the conformational change required in decoding (30). Apparently, the nature of the lower stem plays an important role in both spontaneous and drug-aggravated miscoding and determines the translational accuracy of the mutant decoding sites. Thus, susceptibility of A1555G and C1494T deafness mitoribosomes is the result of two mechanisms, which act in concert: increased drug binding to its target and excessive aggravation of the mutants' inherent deficiency in ribosomal accuracy.

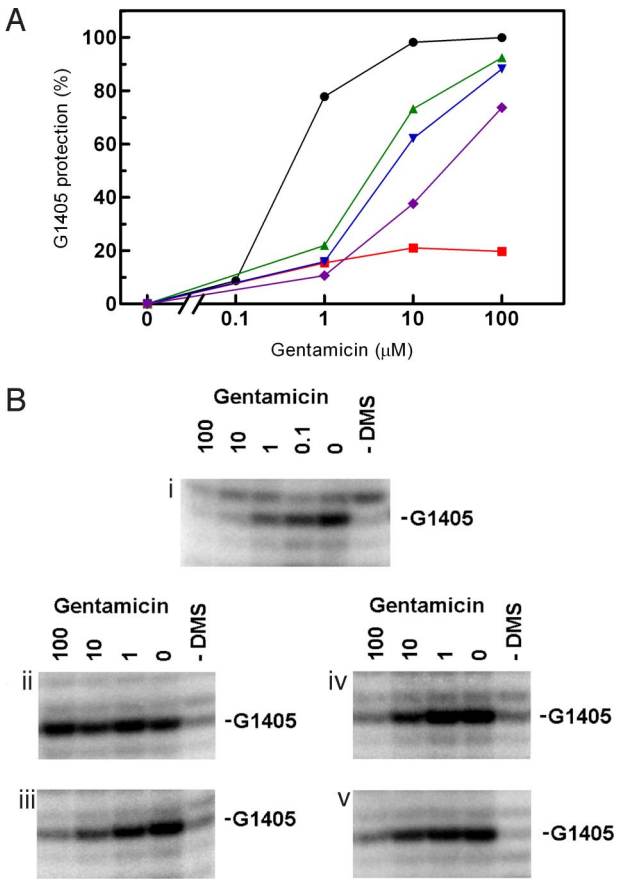


**Fig. 3.** Chemical footprints of gentamicin binding to wild-type and mutant mitochondrial decoding sites in comparison to bacterial wild-type and G1491C ribosomes. (*A*) Gentamicin-dependent protection of G1405 in wild-type mitochondrial (red squares), mutant mt A1555G (green triangles), mutant mt C1494T (blue inverted triangles), and bacterial G1491C (purple diamonds) decoding sites; wild-type bacterial ribosomes (black circles) are included for comparison. (*B*) Corresponding footprinting blots showing primer extensions starting with U1420. *i*, bacterial wild type; *ii*, mt wild type; *iii*, mt A1555G; *iv*, mt C1494T; *v*, bacterial G1491C.

Several lines of evidence link aminoglycoside ototoxicity to the mitochondrial ribosome: (*i*) mitochondrial ribosomes are structurally more similar to their prokaryotic ancestor than to the eukaryotic cytosolic homologues; (*ii*) compared to cytosolic ribosomes, the mitochondrial ribosomes of higher eukaryotes exhibit a remarkable degree of aminoglycoside susceptibility (27) [see Table S2, which compares the drug susceptibility of hybrid bacterial ribosomes with the A site (H44) of human cytosolic ribosomes to that of hybrid ribosomes with the mitochondrial decoding site]; and (*iii*) idiosyncratic drug susceptibility is associated with genetic predisposition, in particular mutations in mtDNA: 20 to 40% of patients with aminoglycoside-induced ototoxicity either carry the A1555G or the C1494T mutation in the 12S rRNA gene (6, 40).

To further assess whether aminoglycoside-induced ototoxicity is a result of the drugs' anti-mitoribosomal activity, we compared the potencies of a series of aminoglycosides to inhibit mitoribosome function with their relative cochleotoxicity in humans (41). The correlation between these two measures (Fig. 4) is consistent with the hypothesis that aminoglycoside-induced cochleotoxicity relates to the drugs' activity against mitochondrial ribosomes. Further evidence for this hypothesis is provided by our finding that netilmicin, which displays the least cochlear toxicity of the clinical aminoglycosides (reviewed in ref. 42), is significantly less active against hybrid mitochondrial ribosomes than gentamicin, tobramycin, or amikacin.

In summary, we provide experimental evidence for a mechanistic linkage between the mitochondrial A1555G and C1494T mutations and hypersusceptibility to aminoglycosides, although the exquisite tissue-specific action of aminoglycoside toxicity (that is, ototoxicity) is likely to involve additional factors (e.g., reactive oxygen species, drug uptake, or polyamine-like activation of NMDA receptors) (2, 43). Our results provide experimental support for aminoglycoside-induced dysfunction of the mitochondrial ribosome. We propose a pathogenic mechanism, in which interference of aminoglycosides with mitochondrial protein synthesis exacerbates the drugs' cochlear toxicity, playing a key role in sporadic dose-dependent and genetically inherited, aminoglycoside-induced deafness. Based upon our experiments, we suggest a scenario of aminoglycoside hearing

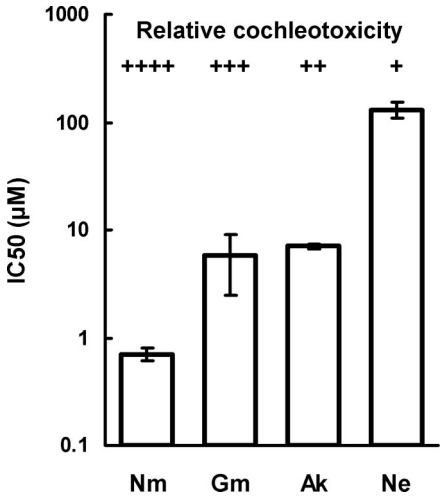


**Fig. 4.** Relationship between inhibition of protein synthesis in mitochondrial hybrid ribosomes and relative *in vitro* cochleotoxicity of aminoglycoside antibiotics. The potencies of a series of cochleotoxic aminoglycosides (Ak, amikacin; Gm, gentamicin; Ne, neamine; Nm, neomycin) in inhibiting protein synthesis in hybrid mitochondrial ribosomes (see Table 3) correlates with the relative cochleotoxicity previously reported by Kotecha and Richardson (41).

loss, which is initiated by mitoribosomal misreading, subsequently via activation of downstream signaling pathways, such as MAPK and JNK (44, 45), misreading results in hair cell death through apoptosis.

## Materials and Methods

**Construction of Mutant Strains with Hybrid Ribosomes.** The recently described *M. smegmatis* mc$^2$ 155 Sm$^5$ Δ*rrnB* (38) was used for all genetic manipulations. Site-directed mutagenesis of its single rRNA operon was done by PCR mutagenesis using hybrid rDNA oligonucleotides comprising the wild-type or mutant mitochondrial helix 44 decoding-site sequence. The resulting hybrid gene fragment was cloned into an integration-proficient plasmid used to transform *M. smegmatis* Δ*rrnB*. Transformants were selected on LB agar plates containing 20 μg/ml paromomycin for gene replacement by homologous recombination. Resulting recombinant *M. smegmatis* cells had the central 34-nucleotide part of the bacterial H44 replaced by its mitochondrial counterpart. Successful replacement of the bacterial decoding-site sequence with the mitochondrial sequence was controlled by sequence analysis of the chromosomal *rrnA* locus.

**Minimal Inhibitory Concentration Assays.** Minimal inhibitory concentrations of neomycin B, paromomycin, kanamycin A, tobramycin, amikacin, gentamicin, netilmicin (all Sigma), and neamine were determined by broth microdilution assays as described previously (46). Neamine was a kind gift of Andrea Vasella, ETH Zurich. The gentamicin used in this study is a mixture of gentamicin $C_1$, gentamicin $C_{1a}$, and gentamicin $C_2$ in a 45:35:30 ratio.

**Isolation and Purification of Ribosomes.** Ribosomes were purified from bacterial cell pellets as described previously (30). In brief, ribosome particles were isolated by successive centrifugations and fractionated by sucrose gradient (10–40%) centrifugation. The 70S ribosome-enriched fraction was pelleted, resuspended in association buffer, incubated for 30 min at 4 °C, dispensed into aliquots, and stored at −80 °C following shock freezing in liquid nitrogen. Ribosome concentrations of 70S were determined by absorption measurements on the basis of 23 pmol ribosomes per $A_{260}$ unit. Integrity and functional activity of purified 70S ribosomes was determined by analytical ultracentrifugation and by assessing their capacity to form initiation complexes, as described previously (29).

**Cell-free AUG(UUU)$_{12}$ Translation Assays.** Cell-free translation reactions were done as described previously (30). A reaction mixture containing *M. smegmatis* tRNA$^{bulk}$, amino acids, S100 extract, energy mix, pyruvate kinase, and polyamines was preincubated with 30 μM [$^{14}$C]-phenylalanine (110 mCi/mmol) and/or 30 μM [$^3$H]-leucine (500 mCi/mmol) at 37 °C for 15 min. The translation reaction was started by addition of ribosomes to a final concentration of 0.25 μM, AUG(UUU)$_{12}$-mRNA (5′-GCGGCAAGGAGGUAAAUA *AUG* (UUU)$_{12}$ *UAA* GCAGG-3′, obtained from Dharmacon) to 1 μM, and aminoglycoside antibiotics in serial dilutions. Following incubation for 60 min at 37 °C, the reaction was stopped by addition of KOH, precipitated polypeptides were collected on filters, and [$^{14}$C]-phenylalanine or [$^3$H]-leucine were quantified. Background values for Phe and Leu incorporation were 0.4 to 0.5 pmol at time zero; the background was not subtracted from the experimental values determined.

**Cell-Free Luciferase Translation Assays.** Purified 70S hybrid ribosomes were used in a coupled transcription-translation reaction as described previously (29). The reaction mixture was incubated for 60 min at 37 °C, stopped on ice, and luciferase assay substrate (Promega) was added. Functional protein was quantified by measuring bioluminescence in a luminometer (Bio-Tek instruments, FLx800).

**Footprinting Analyses.** DMS modification of 70S ribosomes (20 pmol) was performed in 100 μl buffer containing 80 mM potassium cacodylate (pH 6.5), 100 mM ammonium chloride, 20 mM magnesium chloride, 1 mM DTT, and 0.5 mM EDTA. Following ribosome activation for 15 min at 37 °C, gentamicin was added and the reaction mixture was incubated for another 15 min before addition of DMS (6 μl, 1:10 in ethanol). Following a 30-min incubation at 37 °C, the reaction was stopped by addition of 100 μl DMS Stop solution (50 mM Tris, pH 7.5, 300 mM sodium chloride, 1% SDS, 200 mM β-mercaptoethanol). Ribosomes were precipitated with ethanol, pelleted, resuspended in 200 μl 50 mM Tris pH 8, 0.5% SDS, and extracted with phenol/chloroform. DMS-modified RNA was precipitated with ethanol and sodium borohydride reduction and aniline-induced strand scission was performed as described previously (47). Primer extension of 16S rRNA was performed as described (48), using DNA oligonucleotides complementary to 16S-rRNA nucleotides 1445 to 1421. Air-dried gels were scanned and quantified using the STORM PhosphorImaging System with ImageQuant 5.2 Software (Amersham Bioscience).

**ACKNOWLEDGMENTS.** The authors thank Tanja Janušić (Institut für Medizinische Mikrobiologie) for expert technical assistance, Andrea Vasella (Swiss Federal Institute of Technology, Zurich) for kindly providing neamine, and Alexander Mankin for helpful comments on the manuscript. This work was supported by grants from the Swiss National Science Foundation (to E.C.B.) and from the Bonizzi-Theler-Stiftung (to S.N.H.).

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# Supporting Information

Hobbie *et al.* 10.1073/pnas.0811258106

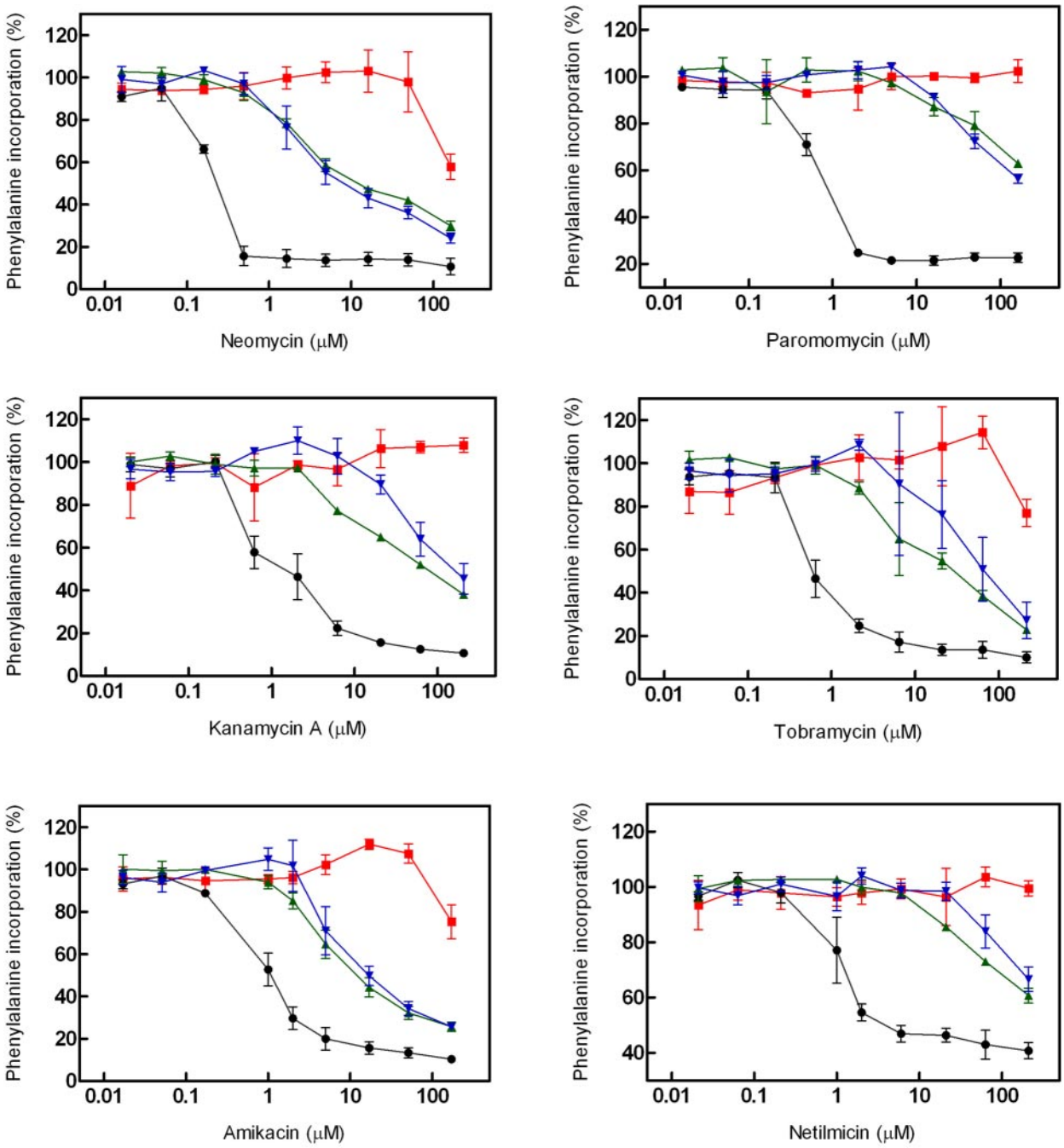


**Fig. S1.** Aminoglycoside susceptibility of bacterial hybrid ribosomes in AUG(UUU)$_{12}$-directed polypeptide synthesis. Dose-response curves of bacterial (black circles), mitochondrial (red squares), mutant A1555G (green triangles), and mutant C1494T (blue inverted triangles) hybrid ribosomes. Corresponding $IC_{50}$ values are given in Table 2.

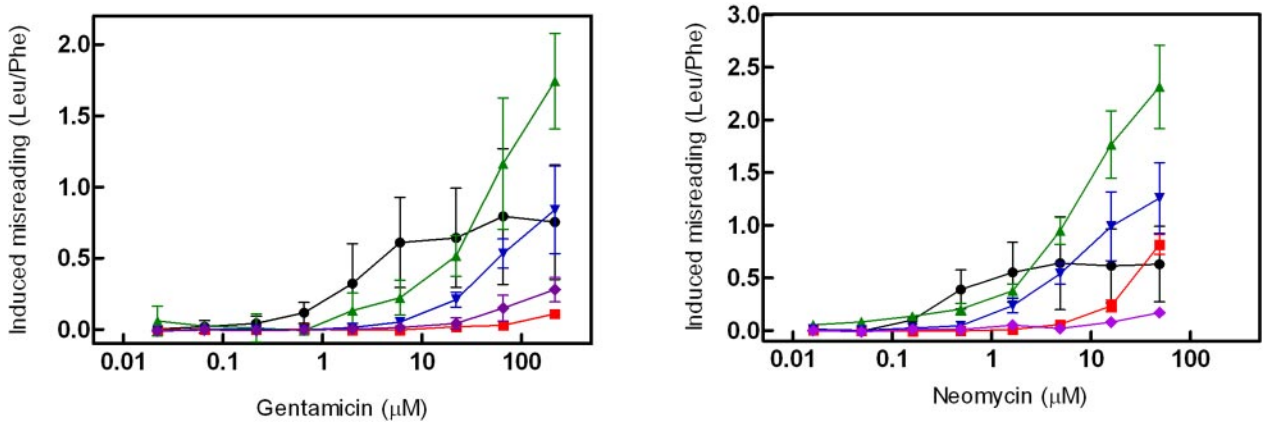


**Fig. S2.** Aminoglycoside-induced miscoding relative to a drug-free control in a *M. smegmatis* G1491C mutant in comparison to mutant mitochondrial decoding sites. Dose-response curves of *M. smegmatis* G1491C (purple diamonds), mitochondrial (red squares), mutant A1555G (green triangles), and mutant C1494T (blue inverted triangles) hybrid ribosomes. Bacterial wild-type ribosomes (black circles) are included for comparison.

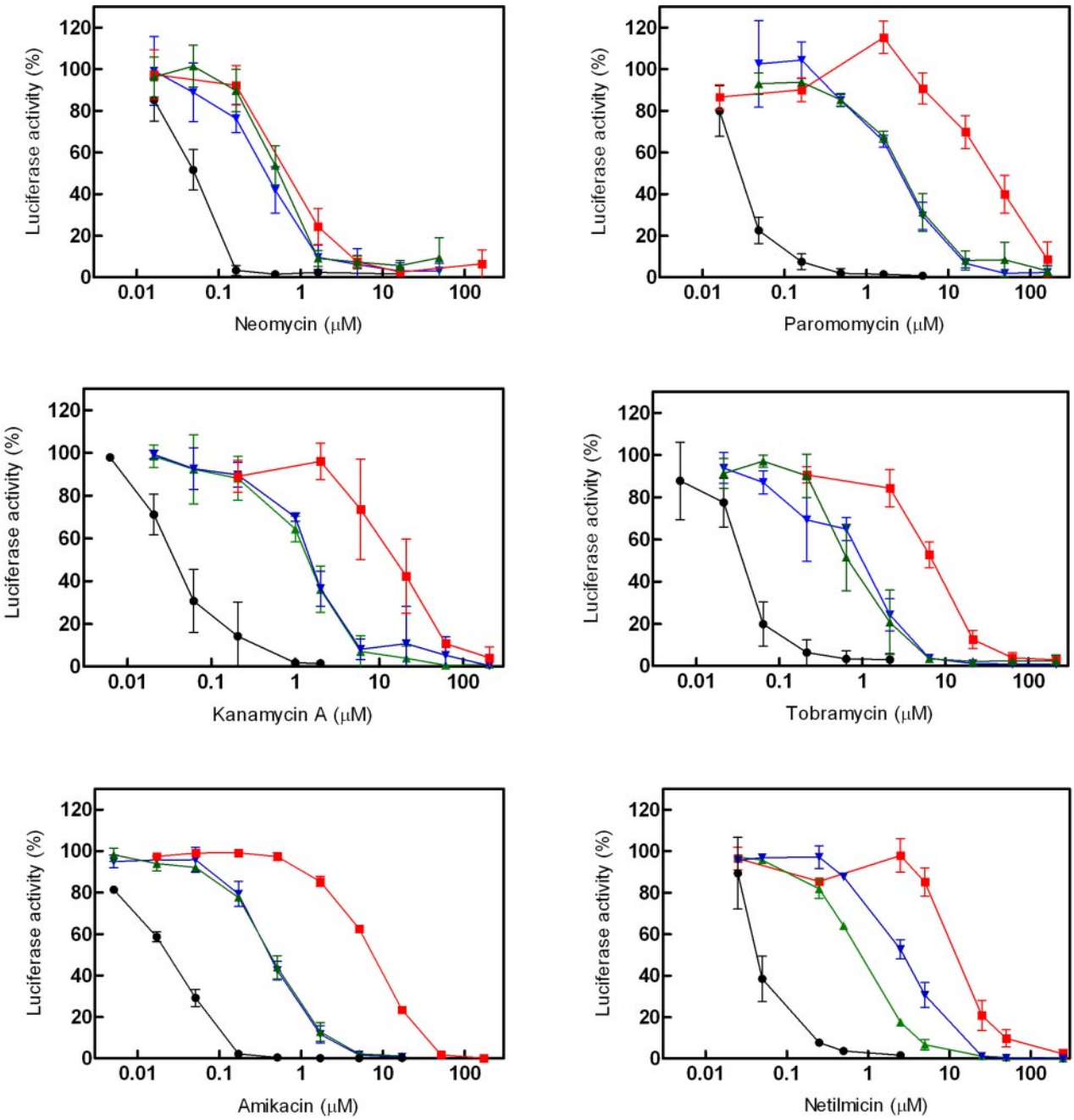


**Fig. S3.** Aminoglycoside-induced inhibition of luciferase synthesis. Dose-response curves of bacterial (black circles), mitochondrial (red squares), mutant A1555G (green triangles), and mutant C1494T (blue inverted triangles) hybrid ribosomes. Corresponding $IC_{50}$ values are given in Table 3.

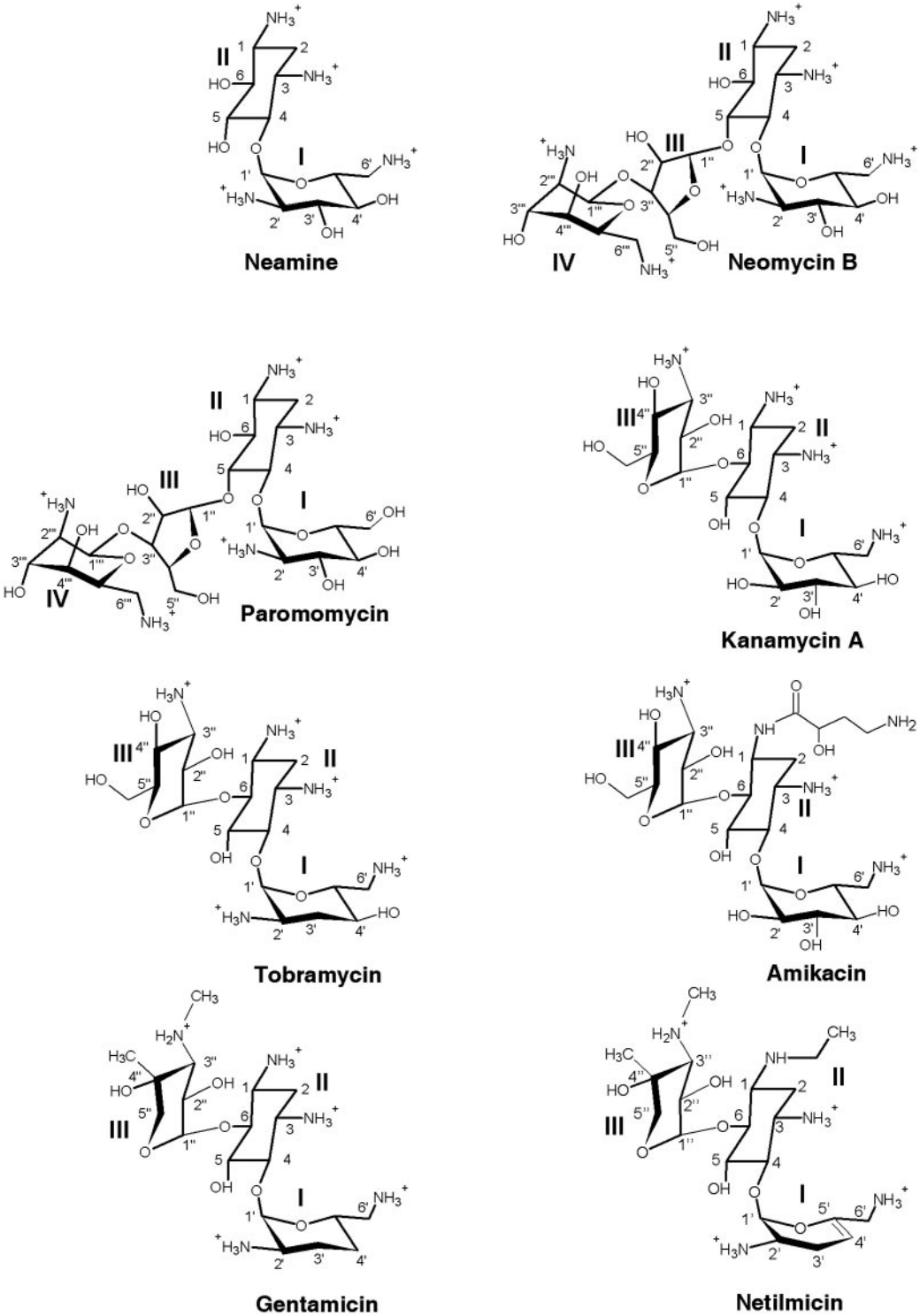


**Fig. S4.** Chemical structures of 2-deoxystreptamine antibiotics used in this study.

**Table S1. Minimal inhibitory concentrations ($\mu$g/ml) against *M. smegmatis* G1491C mutant in comparison to mutant mitochondrial hybrids**

| | A-site rRNA | | | | |
|---|---|---|---|---|---|
| | *M. smegmatis* | Mitochondrial (mt) hybrid | mt A1555G hybrid | mt C1494T hybrid | *M. smegmatis* G1491C |
| Neomycin B | 0.5 | 16–32 | 8 | 8 | 16 |
| Paromomycin | 1 | > 1,024 | 256–512 | 256–512 | 512 |
| Kanamycin A | 1 | 256–512 | 16–32 | 16 | 16–32 |
| Tobramycin | 1 | 128 | 16 | 16 | 16 |
| Amikacin | 0.5 | 32–64 | 2–4 | 2–4 | 4 |
| Gentamicin | 1 | 64–128 | 16–32 | 16–32 | 16–32 |
| Netilmicin | 2 | 512–1,024 | 64 | 64–128 | 128 |

**Table S2. Aminoglycoside susceptibility of the mitochondrial versus the cytosolic decoding site**

| | MIC ($\mu$g/ml) | |
|---|---|---|
| | Mitochondrial hybrid | Cytosolic hybrid |
| Neomycin B | 16–32 | > 1,024 |
| Kanamycin A | 256–512 | > 1,024 |
| Tobramycin | 128 | 1,024 |
| Amikacin | 32–64 | 512–1,024 |
| Gentamicin | 64–128 | > 1,024 |
| Netilmicin | 512–1,024 | > 1,024 |

**(Manuscript in preparation)**

# Minor phylogenetic sequence variations in bacterial rRNA affect species-specific susceptibility to drugs targeting protein synthesis

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** These authors contributed equally to this work.*

**Antibiotics targeting the bacterial ribosome typically bind to highly conserved rRNA regions with only minor phylogenetic sequence variations. By mutagenesis of rRNA sequences in *M. smegmatis* ribosomes we reconstructed the effect of characteristic rRNA residues on drug activity and provide a rationale for differences in species-specific drug susceptibility patterns**.

The bacterial ribosome is target for many antibacterial agents that interfere with protein synthesis, such as aminoglycosides, macrolides, ketolides, oxazolidinones, and lincosamides (1, 29). These compounds target different steps involved in translation including decoding, peptide bond formation and translocation (24, 31, 34). While different classes of antibiotics bind to different ribosomal regions and interfere with different steps of translation, they all interact directly with rRNA nucleotides at or near functionally important sites (32, 33). These rRNA residues typically show high phylogenetic sequence conservation within bacteria. It is unclear whether the minor phylogenetic sequence variations present in the bacterial drug-binding site affect antibiotic susceptibility and/or resistance development. Structures of antibiotics bound to the ribosome have primarily been resolved from extremophilic bacteria such as *Thermus thermophilus, Deinococcus radiodurans* or *Haloarcula morismortui* (5, 11, 28). Most genetic and biochemical data, however, have been generated in *E. coli* and *M. smegmatis* (2, 4, 7).

It has still to be established whether the conclusions drawn from diverse model organisms hold true for other bacterial clades as well. To address this question, we introduced rRNA alterations corresponding to phylogenetic sequence variations that are found in bacteria and which locate to two major drug binding sites, the 23S-rRNA peptidyl transferase region and the 16S-rRNA decoding region. We used a previously described procedure for rRNA

mutagenesis in *M. smegmatis* which results in homogenous populations of mutant ribosomes in an otherwise isogenic background (14). The resulting recombinants were then tested for drug susceptibility by determining minimal inhibitory concentrations, MIC (14).

**Phylogenetic sequence variations in the 23S rRNA peptidyl-transferase region**

Most rRNA nucleotides in the inner peptidyl-transferase region of the large ribosomal subunit are phylogenetically conserved (10). The macrolide/ketolide family of antibiotics binds to a hydrophobic cleft formed by residues 2058, 2059 and 2611 (*E. coli* numbering used throughout) in domain V of 23S-rRNA (see Figure 1) with some drugs making additional contacts in domain II (11, 12, 28). The adenines at 23S-rRNA positions 2058 and 2059 are phylogenetically conserved in bacteria and play an important role in compound binding, emerging of resistance and drug selectivity (1, 3, 27).

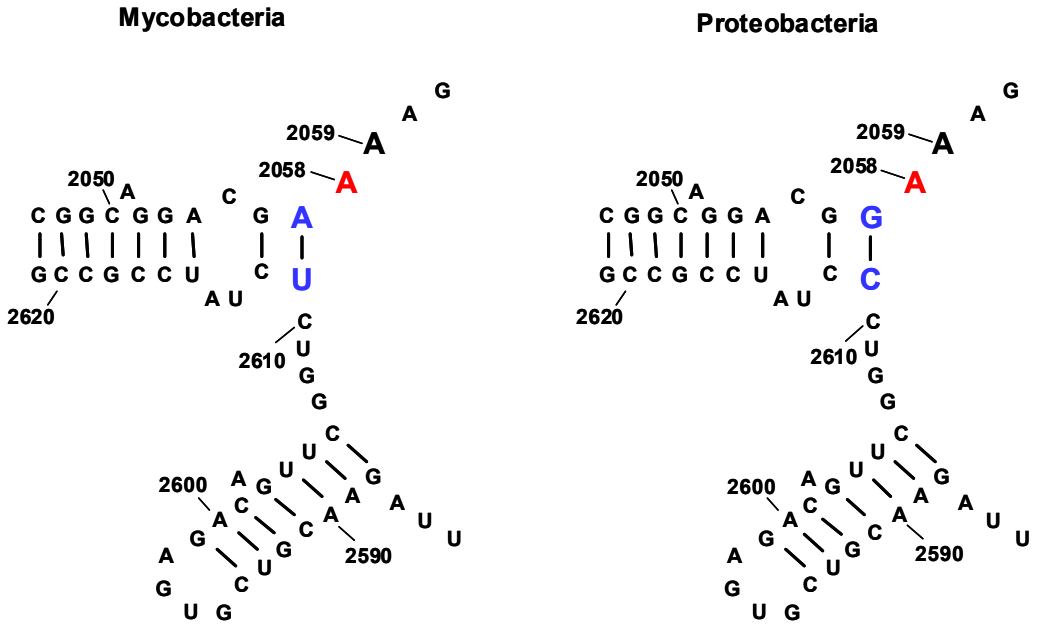


**Figure 1: Secondary structure of domain V of the 23S rRNA. rRNA residues are numbered according to their homologous positions in *E. coli* 23S rRNA. Base pair 2057-2611 is represented in blue and the adenine at position 2058 in red.**

One key interaction appears to be the hydrogen bond between the N1 of A2058 and the 2'-hydroxyl group of the macrolides' desosamine sugar (28, 30). Mutation of A2058 to a guanine (A2058G) greatly impairs the binding of macrolides to ribosomes by both chemical and sterical alteration of the binding site (11, 23). Ribosomal susceptibility to macrolides and ketolides is also determined by proper Watson-Crick base pairing between nucleotides at positions 2057 and 2611 (8), which are typically G-C in Proteobacteria and A-U in Mycobacteria (Figure 1). The respective nature of the 2057-2611 base pair has been shown to affect the resistance phenotype of the A2058G mutation towards ketolides (20). However,

the potential effect of an A2057G-U2611C substitution on ribosomes with an A2058 has remained elusive. Changing the A2057-U2611 base pair in *M. smegmatis* to G2057-C2611 as it is typically found in Proteobacteria had no effect on susceptibility to any of the macrolides or ketolides tested (Table 1). Similarly, the nature of this base pair had no effect on resistance to 14-membered erythromycin and clarithromycin, 15-membered azithromycin and 16-membered spiramycin, tylosin and josamycin macrolides when combined with the A2058G mutation. However, we confirmed that the ketolide-resistance phenotype of the A2058G is indeed dependent on the nature of the 2057-2611 base-pair, as the A2058G mutant is 16-fold more susceptible to telithromycin in the context of a proteobacterial G2057-C2611 sequence than in the context of a mycobacterial A2057-U2611 (Table 1).

| Clade homology | 23S rRNA | | MIC (µg/mL) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 2058 | 2057–2611 | Ery | Cla | Azm | Spm | Tyl | Jsm | Tel |
| Mycobacteria | A | A=U | 8 | 1 | 4-8 | 2 | 2 | 2 | 0.25-0.5 |
| Proteobacteria | A | G≡C | 8-16 | 1 | 2-4 | 1 | 2 | 1 | 0.25-0.5 |
| Mycobacteria | G | A=U | > 512 | > 512 | > 512 | 128 | 8 | 8 | 128 |
| Proteobacteria | G | G≡C | > 512 | > 512 | > 512 | 64 | 4 | 8 | 8 |

**Table 1: Minimal inhibitory concentrations of various macrolides/ketolides in *M. smegmatis* 23S rRNA variants. Ery: erythromycin; Cla: clarithromycin; Azm: azithromycin; Spm: spiramycin; Tyl: tylosin; Jsm: josamycin; Tel: telithromycin**

**Phylogenetic sequence variations in 16S rRNA helix 44**

Nucleotides of 16S-rRNA helices 18, 34 and 44 form the core aminoacyl tRNA acceptor site (A-site) and are highly conserved (9), reflecting their importance in mRNA decoding. Aminoglycoside antibiotics bind to the A-site by direct contacts to helix 44 and induce misreading of the genetic code (5, 6). While aminoglycosides form a number of hydrogen bonds with different nucleotides in helix 44, their interactions with rRNA residues 1408, 1409 and 1491 (*E. coli* numbering) appear to be most critical for drug binding (14, 16, 17, 21).

16S-rRNA residue 1408 is an adenine in all bacteria (Figure 2). Among all A-site mutations that confer aminoglycoside resistance, the 1408 adenine to guanine mutation is the predominant alteration in clinical drug resistant strains (25, 26). This transition mutation alone is sufficient to impart high level resistance to 6'-$NH_2$ aminoglycosides (Table 2), by disrupting

the interaction between A1408 and the compound's ring 1 aminosugar (22). It is also believed to function as the main specificity determinant of aminoglycosides, because higher eukaryotes carry a guanine at this position (3).

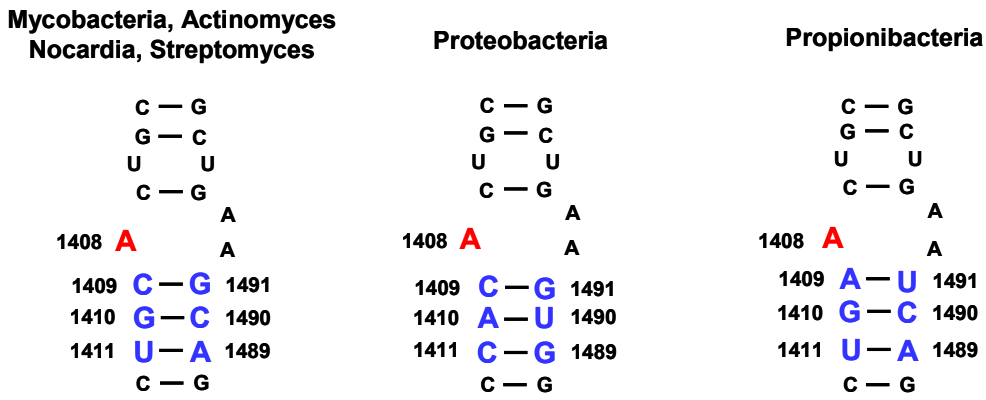


**Figure 2: Secondary structure of 16S rRNA helix 44 decoding site. rRNA residues are numbered according to their homologous positions in *E. coli* 16S rRNA. Phylogenetic sequence variations analyzed in this study are represented in blue, the adenine at position 1408 in red.**

Minor sequence variations occur within the aminoglycoside-binding pocket of helix 44 involving 16S-rRNA base-pair 1409-1491(9). The majority of eubacteria are characterized by a 1409 pyrimidine-1491 purine (C-G) interaction, compared to Propionibacteria which carry a 1409 purine-1491 pyrimidine (A-U) base pair (Figure 2). Substituting the C-G base pair in *M. smegmatis* with a propionibacterial A-U considerably reduced susceptibility to all 4,5 and 4,6 aminoglycosides tested (Table 2). This observation is in agreement with the lower aminoglycoside susceptibility of Propionibacteria (18); amikacin is the least affected because its L-haba group interacts with additional nucleotides within helix 44 and stabilizes drug binding (19). Combining the 1409A-1491U interaction further with an A1408G alteration resulted in high-level resistance to all aminoglycosides including paromomycin, a 6'-OH substituted 4,5 aminoglycoside (Table 2). The high-level resistance phenotype to paromomycin is apparently a combined effect of perturbing contacts to both G1491 and A1408, which would be in agreement with previous data demonstrating that U1491 increased resistance towards 6'-OH aminoglycosides such as paromomycin (21).

To study the contribution of a 1409-1491 purine/pyrimidine switch to aminoglycoside susceptibility in the presence of a wt 1408A in more detail we introduced base pairs G-C and U-A. Base pair 1409U-1491A is a pyrimidine-purine interaction similar to the C-G found in the majority of eubacteria. Introduction of the U-A base-pair decreased susceptibility to 4,5-aminoglycosides, in particular to paromomycin, but had virtually no effect on susceptibility to 4,6-compounds (Table 2). Introduction of a purine-pyrimidine G-C interaction similar to the A-U found in Propionibacteria, with the exception of amikacin, however, resulted in significant

resistance to 4,5-and 4,6-aminoglycosides with the 4,5-compounds being more affected (Table 2). In line with our previous investigations and the different orientiaton of the aminoglycosides' sugars linked to position 5 or 6 of the neamine core (20), these results demonstrate that in general the 4,5-compounds and in particular the 6'-OH paromomycin are more dependent on a proper 1409-1491 interaction than the 4,6-compounds. From our data we infer that in the presence of a 1408A there is a gradient of susceptibility for the 1409-1491 interaction, i.e. C-G > U-A > G-C > A-U indicating that both the purine/pyrimidine interaction and the specific nucleotide are relevant.

| Clade homology | 16S rRNA residues | | | | MIC (µg/mL) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1408 | 1409–1491 | 1410–1490 | 1411–1489 | 6'-OH | 6'-$NH_2$ | | | | |
| | | | | | Pm | Nm | Gm | Tb | Km | Ak |
| Actinomycetales | A | C≡G | G≡C | U=A | 1 | 0.5 | 1 | 1 | 1 | 0.5 |
| Proteobacteria | A | C≡G | A=U | C≡G | 1 | 0.5 | 0.5-1 | 0.5-1 | 0.5 | 0.5 |
| Propionibact. | A | A=U | G≡C | U=A | 64 | 8-16 | 16 | 32 | 16 | 2 |
| - | A | U=A | G≡C | U=A | 8-16 | 1-2 | 1 | 2 | 1 | 0.5 |
| - | A | G≡C | G≡C | U=A | 32 | 2-4 | 4 | 4 | 2 | 0.5 |
| Actinomycetales | G | C≡G | G≡C | U=A | 64 | > 1024 | > 1024 | > 1024 | > 1024 | > 1024 |
| Proteobacteria | G | C≡G | A=U | C≡G | 64 | > 1024 | > 1024 | 1024 | > 1024 | > 1024 |
| Propionibacteria | G | A=U | G≡C | U=A | > 1024 | > 1024 | > 1024 | > 1024 | > 1024 | > 1024 |

**Table 2: Minimal inhibitory concentrations of various aminoglycosides in *M. smegmatis* 16S rRNA variants. Pm, paromomycin; Nm, neomycin; Gm, gentamicin; Tb, tobramycin; Km, kanamycin A; Ak, amikacin**

Previously, limitations in genetic manipulation did not allow studying the effect of the bacterial 1409-1491 polymorphism on aminoglycoside susceptibility in isogenic mutants. Rather, investigations were limited to testing different bacterial species representative of the corresponding sequence polymorphism. In these early studies it was concluded that the C1409-G1491 to A1409-U1491 polymorphism is not associated with resistance (21). Using more recently developed genetic techniques we have now been able to refine this statement and to define the role of the 1409-1491 base-pairing polymorphism in aminoglycoside susceptibility more precisely.

We have recently found that the 16S rRNA interaction 1410-1490 may have a subtle influence on aminoglycoside susceptibility in ribosomes with a non Watson-Crick 1409-1491

interaction, e.g. 1409C-1491C (15). In the current study, base pair variations in 1410-1490 did not affect aminoglycoside susceptibility (Table 2). We conclude that in the presence of a 1409-1491 base-pairing, the nature of 1410-1490 Watson-Crick base pair does not affect aminoglycoside susceptibility.

The ribosome is target for many different classes of antibiotic compounds (13, 24). Minor structural differences, such as species-specific compositions of the drug binding site may affect susceptibility and resistance to drugs interfering with bacterial protein synthesis. From our results we conclude that natural sequence variations in the ribosomal PTC of bacteria do not affect drug susceptibility, but impact on the resistance phenotype of the A2058G mutation, in particular to the ketolide telithromycin. In contrast, natural sequence variations in the ribosomal A site of bacteria affect species-specific drug susceptibility, but do not impact on the resistance phenotype of the A1408G mutation to 6'-$NH_2$ aminoglycosides. In the past decade, atomic resolution structures of numerous ribosome-drug complexes have become available (34). A multi-pronged approach combining genetics, biochemistry and structural data should facilitate the modification of existing and evaluation of newer anti-microbial drugs.

The authors thank Tanja Janušić (Institut für Medizinische Mikrobiologie) for expert technical assistance. This work was supported in part by the University of Zürich

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## Personal contributions

My personal contributions to the various projects in the results is as follows

**Project 1 - Mitochondrial deafness alleles confer misreading of the genetic code**
Sven N. Hobbie, Christian M. Bruell, **Subramanian Akshay**, Sarath K. Kalapala, Dmitry Shcherbakov, and Erik C. Böttger

- Generated $(UUU)_{12}$ based data of phenylalanine and leucine incorporation for the A1555G and C1494U mutants in comparison to the mitochondrial hybrid and the *M. smegmatis* wild-type.
- Generated relative luciferase activity vs leucine incorporation data for the A1555G in comparison to the mitochondrial hybrid and *M. smegmatis* wild-type.
- Generated tobramycin-induced misreading data in the $(UUU)_{12}$ based assay for the A1555G and C1494U mutants in comparison to the mitochondrial hybrid and the *M. smegmatis* wild-type.
- Participated in manuscript writing.

**Project 2 - Genetic analysis of interactions with eukaryotic rRNA identify the mitoribosome as target in aminoglycoside ototoxicity**
Sven N. Hobbie*, **Subramanian Akshay***, Sarath K. Kalapala*, Christian M. Bruell, Dmitry Shcherbakov, and Erik C. Böttger

* *Equal contribution*

- Generated drug-induced inhibition data to calculate the $IC_{50}$ values and misreading data in the $(UUU)_{12}$ based assay for the A1555G and C1494U mutants in comparison to the mitochondrial hybrid and the *M. smegmatis* wild-type.
- Generated drug-induced inhibition data in the TnT based luciferase assay for the A1555G and C1494U mutants in comparison to the mitochondrial hybrid and the *M. smegmatis* wild-type to calculate the $IC_{50}$ values.

**Project 3 – Minor phylogenetic sequence variations in the bacterial rRNA affect species-specific susceptibility to drugs targeting protein synthesis**
**Subramanian Akshay***, Mihai Bertea*, Sven N. Hobbie*, Björn Oettinghaus, Rashid Akbergenov, and Erik C. Böttger

* *Equal contribution*

- Prepared A1408G/C1409A/G1491U mutant strain.
- Performed Minimal inhibitory concentration (MIC) assays.
- Wrote the manuscript together with SNH, RA and ECB

## 3. Publications

1. S.N. Hobbie, C. Bruell, S. Kalapala, **S. Akshay**, S. Schmidt, P. Pfister, and E. C. Böttger. 2006. A genetic model to investigate drug-target interactions at the ribosomal decoding site. Biochimie 88:1033-43.
2. S.N. Hobbie*, S. K. Kalapala*, **S. Akshay***, C. Bruell, S. Schmidt, S. Dabow, A. Vasella, P. Sander, and E. C. Bottger. 2007. Engineering the rRNA decoding site of eukaryotic cytosolic ribosomes in bacteria. Nucleic Acids Res. 35:6086-6093.
   * *equal contribution*
3. S.N. Hobbie, C. M. Bruell, **S. Akshay**, S. K. Kalapala, D. Shcherbakov, and E. C. Böttger. 2008. Mitochondrial deafness alleles confer misreading of the genetic code. Proc. Natl. Acad. Sci. U S A 105:3244-3249.
4. S.N. Hobbie*, **S. Akshay***, S. K. Kalapala*, C. M. Bruell, D. Shcherbakov, and E. C. Böttger. 2008. Genetic analysis of interactions with eukaryotic rRNA identify the mitoribosome as target in aminoglycoside ototoxicity. Proc Natl Acad Sci U S A 105:20888-93.
   * *equal contribution*
5. **S. Akshay***, M. Bertea*, S.N. Hobbie*, B. Oettinghaus, R. Akbergenov, and E.C. Böttger. Minor phylogenetic sequence variations in bacterial rRNA affect species-specific susceptibility to drugs targeting protein synthesis (manuscript in preparation)
   * *equal contribution*

## 4. Conference presentations

2006 2nd FEMS Congress of European Microbiologists, Madrid, Spain
*Genetic analysis of aminoglycoside binding to the ribosomal decoding site*
S.N. Hobbie*, S. Kalapala, **S. Akshay**, B. Francois, E. Westhof, E.C. Böttger

2007 EMBO Conference on Protein Synthesis and Translational Control, Heidelberg, Germany
*Transplanting human and protozoan decoding sites into bacterial hybrid ribosomes*
S.N. Hobbie*, S. Kalapala, **S. Akshay**, E.C. Böttger
*A molecular basis for aminoglycoside-induced ototoxicity*
**S. Akshay***, C. Brüll, S.N. Hobbie, E.C. Böttger

Ribosomes: Form and Function, Cape Cod, Massachusetts, USA
*Engineering bacterial protein synthesis machinery for mitochondrial decoding: deafness alleles confer misreading of the genetic code.*
E.C. Böttger*, C. Brüll, **S. Akshay**, S. Kalapala, S.N. Hobbie

2008 60th Annual Meeting fo the Deutsche Gesellschaft für Hygiene und Mikrobiologie, Göttingen, Germany
*Aminoglycosides: Structural analyses of interactions with eukaryotic A-sites point to the mitoribosome as target and identify mechanisms of toxicity*
S.N. Hobbie*, **S. Akshay**, S. Kalapala, C. Brüll, D. Scherbakov, E.C. Böttger

EMBO RNA and Disease: RNA Metabolism and Associated Pathologies, Rome, Italy
*Mitochondrial 12S rRNA mutation increase ribosomal susceptibility to aminoglycoside-induced mistranslation*
D. Scherbakov*, S.N. Hobbie, S. Kalapala, **S. Akshay**, E.C. Böttger

67th Annual Assembly of the Swiss Society for Microbiology, Interlaken, Switzerland
*Ribosomal mutation A1408G does not affect streptomycin susceptibility*
**S. Akshay***, S.N. Hobbie, E.C. Böttger
*Transplanting eukaryotic decoding sites into bacterial hybrid ribosomes*
S. Kalapala*, **S. Akshay**, C. Brüll, S.N. Hobbie, E.C. Böttger

48th Annual ICAAC/ IDSA 46th Annual Meeting, Washington D.C. USA
*Aminoglycosides: Structural analyses of interactions with eukaryotic A-sites point to the mitoribosome as target and identify mechanisms of toxicity*
S.N. Hobbie*, **S. Akshay**, S. Kalapala, C. Brüll, D. Scherbakov, E.C. Böttger

International Symposium on Mitochondrial Physiology and Pathology, Bari, Italy
*Mitochondrial deafness alleles affect the accuracy of mRNA translation*
S.N. Hobbie*, **S. Akshay**, S. Kalapala, C. Brüll, D. Scherbakov, E.C. Böttger
*Aminoglycosides and ototoxicity: Structural analyses of interactions with eukaryotic A-sites identify the mitoribosome as drug target*
S.N. Hobbie, **S. Akshay**, S. Kalapala, C. Brüll, D. Scherbakov, E.C. Böttger*

13th Annual Meeting of the RNA society, Berlin, Germany
*Aminoglycosides differentially affect protein synthesis in bacterial hybrid ribosomes carrying various versions of the human decoding site: the role of mitochondrial ribosome in drug toxicity*
**S. Akshay***, C. Brüll, S.N. Hobbie, E.C. Böttger

2009 68th Annual Assembly of the Swiss Society for Microbiology, Lausanne, Switzerland
*Aminoglycosides: Structural analyses of interactions with eukaryotic A-sites point to the mitoribosome as target and identify mechanisms of toxicity*

D. Scherbakov*, **S. Akshay**, S. Kalapala, M. Kulstrunk, R. Akbergenov, E.C. Böttger

8th International Conference on Ribosome Synthesis, Regensburg, Germany
*12S rRNA deafness alleles and aminoglycoside induced ototoxicity converge on mitochondrial mistranslation as mechanism of disease pathogenesis*
**S. Akshay***, S. Kalapala, R. Akbergenov, D. Scherbakov, E.C. Böttger
*Probing of aminoglycoside interaction with the ribosmal decoding A-site by site-directed mutagenesis*
S. Kalapala*, **S. Akshay**, D. Scherbakov, R. Akbergenov, E.C. Böttger

EMBO Conference on Protein Synthesis and Translational Control, Heidelberg, Germany
*rRNA sequence polymorphism within the bacterial domain and susceptibility to drugs targeting protein synthesis*
R. Akbergenov*, **S. Akshay**, S. Kalapala, M. Bertea, M. Kulstrunk, E.C. Böttger

2010 Ribosomes 2010, Orvieto, Italy
*rRNA sequence polymorphism within the bacterial domain and susceptibility to drugs targeting protein synthesis*
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**2000-2002 –** Masters of Science in Plant Molecular Biology from University of Delhi. Title of the M.Sc. thesis: "*Isolation and characterization of a putative methylated DNA binding protein from Arabidopsis thaliana*".

**2002-2004 –** Junior research fellow at the Center for Plant Molecular Biology, University of Delhi. Title of the project: *"Cloning of fruit ripening related genes from tomato and study of their expression".*

**2004-2005 –** Institute for Molecular Biology and Biophysics, ETH Zürich.

**2005-present –** Doctoral studies at the Institute of Medical Microbiology, University of Zürich under the supervision of Prof. Dr. Erik C. Böttger. Title of the Ph.D. thesis *"Sequence variations in the ribosomal decoding and peptidyl transferase sites determine sensitivity to aminoglycosides and macrolides".*

LIST OF PUBLICATIONS:

1. S.N. Hobbie, C. Bruell, S. Kalapala, **S. Akshay**, S. Schmidt, P. Pfister, and E. C. Böttger. 2006. A genetic model to investigate drug-target interactions at the ribosomal decoding site. **Biochimie** 88:1033-43.
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3. S.N. Hobbie, C. M. Bruell, **S. Akshay**, S. K. Kalapala, D. Shcherbakov, and E. C. Böttger. 2008. Mitochondrial deafness alleles confer misreading of the genetic code. **Proc. Natl. Acad. Sci. U S A** 105:3244-3249.
4. S.N. Hobbie*, **S. Akshay***, S. K. Kalapala*, C. M. Bruell, D. Shcherbakov, and E. C. Böttger. 2008. Genetic analysis of interactions with eukaryotic rRNA identify the mitoribosome as target in aminoglycoside ototoxicity. **Proc Natl Acad Sci U S A** 105:20888-93.
   * *equal contribution*
5. **S. Akshay***, M. Bertea*, S.N. Hobbie*, B. Oettinghaus, R. Akbergenov, and E.C. Böttger. Minor phylogenetic sequence variations in bacterial rRNA affect species-specific susceptibility to drugs targeting protein synthesis (manuscript in preparation)

   * *equal contribution*